

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau247  
29

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : C12N 9/00		A2	(11) International Publication Number: WO 99/49019
			(43) International Publication Date: 30 September 1999 (30.09.99)
(21) International Application Number: PCT/US99/06698		(72) Inventor; and	
(22) International Filing Date: 26 March 1999 (26.03.99)		(75) Inventor/Applicant (for US only): SZENT-GYORGYI, Christopher [US/US]; 719 Duncan Avenue, Pittsburgh, PA 15237 (US).	
(30) Priority Data:		(74) Agent: SEIDMAN, Stephanie, L.; Heller Ehrman White & McAuliffe, Suite 700, 4250 Executive Square, La Jolla, CA 92037 (US).	
60/079,624 27 March 1998 (27.03.98) US			
60/089,367 15 June 1998 (15.06.98) US			
60/102,939 1 October 1998 (01.10.98) US			
(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
US 60/079,624 (CIP)			
Filed on 27 March 1998 (27.03.98)			
US 60/089,367 (CIP)			
Filed on 15 June 1998 (15.06.98)			
US 60/102,939 (CIP)			
Filed on 1 October 1998 (01.10.98)			
(71) Applicant (for all designated States except US): PROLUME, LTD. [US/US]; 1085 William Pitt Way, Pittsburgh, PA 15236 (US).		Published	
(71)(72) Applicant and Inventor: BRYAN, Bruce, J. [US/US]; 716 N. Arden Drive, Beverly Hills, CA 90210 (US).		Without international search report and to be republished upon receipt of that report.	
(54) Title: LUCIFERASES, FLUORESCENT PROTEINS, NUCLEIC ACIDS ENCODING THE LUCIFERASES AND FLUORESCENT PROTEINS AND THE USE THEREOF IN DIAGNOSTICS, HIGH THROUGHPUT SCREENING AND NOVELTY ITEMS			
(57) Abstract			
<p>Isolated and purified nucleic acid molecules that encode a luciferase from <i>Renilla mulleri</i>, <i>Gaussia</i> and <i>Pleuromamma</i>, and the proteins encoded thereby are provided. Isolated and purified nucleic acids encoding green fluorescent proteins from the genus <i>Renilla</i> and <i>Ptilosarcus</i>, and the green fluorescent proteins encoded thereby are also provided. Compositions and combinations comprising the green fluorescent proteins and/or the luciferase are further provided.</p>			

BEST AVAILABLE COPY

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KC	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

**LUCIFERASES, FLUORESCENT PROTEINS, NUCLEIC ACIDS ENCODING THE  
LUCIFERASES AND FLUORESCENT PROTEINS AND THE USE THEREOF IN  
DIAGNOSTICS, HIGH THROUGHPUT SCREENING AND NOVELTY ITEMS**

**RELATED APPLICATIONS**

- 5           This application claims priority to U.S. provisional application Serial No. 60/102,939, filed October 1, 1998, to Bruce Bryan and Christopher Szent-Gyorgyi, entitled "LUCIFERASES, FLUORESCENT PROTEINS, NUCLEIC ACIDS ENCODING THE LUCIFERASES AND FLUORESCENT PROTEINS AND THE USE THEREOF IN DIAGNOSTICS, HIGH THROUGHPUT SCREENING AND NOVELTY  
10 ITEMS". Priority is also claimed to U.S. provisional application Serial No. 60/089,367, filed June 15, 1998, to Bruce Bryan and Christopher Szent-Gyorgyi, entitled "GAUSSIA LUCIFERASE, NUCLEIC ACIDS ENCODING THE LUCIFERASE AND METHODS USING THE LUCIFERASE", and to U.S. provisional application Serial No. 60/079,624, filed March 27, 1998, to Bruce  
15 Bryan and Christopher Szent-Gyorgyi, entitled "RENILLA GREEN FLUORESCENT PROTEIN COMPOSITIONS AND METHODS." For U.S. purposes, benefit of priority to each of these applications is claimed under 35 U.S.C. § 119(e).

- This application is also related to subject matter in U.S. application Serial No. 08/757,046, filed November 25, 1996, to Bruce Bryan entitled  
20 "BIOLUMINESCENT NOVELTY ITEMS", now U.S. Patent No. 5,876,995, issued March 2, 1999, and in U.S. application Serial No. 08/597,274, filed February 6, 1996, to Bruce Bryan, entitled "BIOLUMINESCENT NOVELTY ITEMS". This application is also related to U.S. application Serial No. 08/908,909, filed August 8, 1997, to Bruce Bryan entitled "DETECTION AND VISUALIZATION OF  
25 NEOPLASTIC TISSUE AND OTHER TISSUES". The application is also related to U.S. application Serial No. 08/990,103, filed December 12, 1997, to Bruce Bryan entitled "APPARATUS AND METHODS FOR DETECTING AND IDENTIFYING INFECTIOUS AGENTS".

- The subject matter of each of the above noted U.S. applications and  
30 provisional applications is herein incorporated by reference in its entirety.

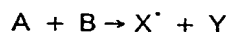
-2-

**FIELD OF INVENTION**

The present invention relates to isolated and purified nucleic acids and encoded proteins from the genera *Renilla*, *Gaussia*, *Philocarpus* and *Pleuromamma*. More particularly, nucleic acids encoding luciferase and  
5 fluorescent proteins from species of these genera are provided.

**BACKGROUND OF THE INVENTION**

Luminescence is a phenomenon in which energy is specifically channeled to a molecule to produce an excited state. Return to a lower energy state is accompanied by release of a photon (hy). Luminescence includes fluorescence,  
10 phosphorescence, chemiluminescence and bioluminescence. Bioluminescence is the process by which living organisms emit light that is visible to other organisms. Luminescence may be represented as follows:



15 where  $X^*$  is an electronically excited molecule and  $h\nu$  represents light emission upon return of  $X^*$  to a lower energy state. Where the luminescence is bioluminescence, creation of the excited state derives from an enzyme catalyzed reaction. The color of the emitted light in a bioluminescent (or chemiluminescent or other luminescent) reaction is characteristic of the excited  
20 molecule, and is independent from its source of excitation and temperature.

An essential condition for bioluminescence is the use of molecular oxygen, either bound or free in the presence of a luciferase. Luciferases, are oxygenases, that act on a substrate, luciferin, in the presence of molecular oxygen and transform the substrate to an excited state. Upon return to a lower  
25 energy level, energy is released in the form of light [for reviews see, e.g., McElroy et al. (1966) in *Molecular Architecture in Cell Physiology*, Hayashi et al., eds., Prentice-Hall, Inc., Englewood Cliffs, NJ, pp. 63-80; Ward et al., Chapter 7 in *Chemi-and Bioluminescence*, Burr, ed., Marcel Dekker, Inc. NY, pp.321-358; Hastings, J. W. in (1995) *Cell Physiology:Source Book*, N. Sperelakis (ed.), Academic Press, pp 665-681; *Luminescence, Narcosis and Life in the Deep Sea*, Johnson, Vantage Press, NY, see, esp. pp. 50-56].  
30



-3-

Though rare overall, bioluminescence is more common in marine organisms than in terrestrial organisms. Bioluminescence has developed from as many as thirty evolutionarily distinct origins and, thus, is manifested in a variety of ways so that the biochemical and physiological mechanisms responsible for bioluminescence in different organisms are distinct. Bioluminescent species span many genera and include microscopic organisms, such as bacteria [primarily marine bacteria including *Vibrio* species], fungi, algae and dinoflagellates, to marine organisms, including arthropods, mollusks, echinoderms, and chordates, and terrestrial organism including annelid worms and insects.

#### Assays employing bioluminescence

During the past twenty years, high-sensitivity biochemical assays used in research and in medicine have increasingly employed luminescence and fluorescence rather than radioisotopes. This change has been driven partly by the increasing expense of radioisotope disposal and partly by the need to find more rapid and convenient assay methods. More recently, the need to perform biochemical assays *in situ* in living cells and whole animals has driven researchers toward protein-based luminescence and fluorescence. The uses of firefly luciferase for ATP assays, aequorin and obelin as calcium reporters, *Vargula* luciferase as a neurophysiological indicator, and the *Aequorea* green fluorescent protein as a protein tracer and pH indicator show the potential of bioluminescence-based methods in research laboratories.

Bioluminescence is also beginning to directly impact medicine and biotechnology; for example, *Aequorea* GFP is employed to mark cells in murine model systems and as a reporter in high throughput drug screening. *Renilla* luciferase is under development for use in diagnostic platforms.

#### Bioluminescence generating systems

Bioluminescence, as well as other types of chemiluminescence, is used for quantitative determinations of specific substances in biology and medicine. For example, luciferase genes have been cloned and exploited as reporter genes in numerous assays, for many purposes. Since the different luciferase systems have different specific requirements, they may be used to detect and quantify a

-4-

variety of substances. The majority of commercial bioluminescence applications are based on firefly [*Photinus pyralis*] luciferase. One of the first and still widely used assays involves the use of firefly luciferase to detect the presence of ATP. It is also used to detect and quantify other substrates or co-factors in the  
5 reaction. Any reaction that produces or utilizes NAD(H), NADP(H) or long chain aldehyde, either directly or indirectly, can be coupled to the light-emitting reaction of bacterial luciferase.

Another luciferase system that has been used commercially for analytical purposes is the *Aequorin* system. The purified jellyfish photoprotein, aequorin,  
10 is used to detect and quantify intracellular  $\text{Ca}^{2+}$  and its changes under various experimental conditions. The *Aequorin* photoprotein is relatively small [ $\sim 20\text{kDa}$ ], nontoxic, and can be injected into cells in quantities adequate to detect calcium over a large concentration range [ $3 \times 10^{-7}$  to  $10^{-4}$  M].

Because of their analytical utility, luciferases and substrates have been  
15 studied and well-characterized and are commercially available [e.g., firefly luciferase is available from Sigma, St. Louis, MO, and Boehringer Mannheim Biochemicals, Indianapolis, IN; recombinantly produced firefly luciferase and other reagents based on this gene or for use with this protein are available from Promega Corporation, Madison, WI; the aequorin photoprotein luciferase from  
20 jellyfish and luciferase from *Renilla* are commercially available from Sealite Sciences, Bogart, GA; coelenterazine, the naturally-occurring substrate for these luciferases, is available from Molecular Probes, Eugene, OR]. These luciferases and related reagents are used as reagents for diagnostics, quality control, environmental testing and other such analyses.

25 Because of the utility of luciferases as reagents in analytical systems and the potential for use in high throughput screening systems, there is a need to identify and isolated a variety of luciferases that have improved or different spectral properties compared to those presently available. For all these reasons, it would be advantageous to have luciferases from a variety of species, such as  
30 *Gaussia* and various *Renilla* species available.

-5-

### Fluorescent Proteins

Reporter genes, when co-transfected into recipient cells with a gene of interest, provide a means to detect transfection and other events. Among reporter genes are those that encode fluorescent proteins. The

5 bioluminescence generating systems described herein are among those used as reporter genes. To increase the sensitivity bioluminescence generating systems have been combined with fluorescent compounds and proteins, such as naturally fluorescent phycobiliproteins. Also of interest are the fluorescent

10 proteins that are present in a variety of marine invertebrates, such as the green and blue fluorescent proteins, particularly the green fluorescent protein (GFP) of *Aequorea victoria*.

The green fluorescent proteins (GFP) constitute a class of chromoproteins found only among certain bioluminescent coelenterates. These accessory proteins are fluorescent and function as the ultimate bioluminescence

15 emitter in these organisms by accepting energy from enzyme-bound, excited-state oxyluciferin (e.g., see Ward *et al.* (1979) J. Biol. Chem. 254:781-788; Ward *et al.* (1978) Photochem. Photobiol. 27:389-396; Ward *et al.* (1982) Biochemistry 21:4535-4540).

The best characterized GFPs are those isolated from the jellyfish species

20 *Aequorea*, particularly *Aequorea victoria* (*A. victoria*) and *Aequorea forskålea* (Ward *et al.* (1982) Biochemistry 21:4535-4540; Prendergast *et al.* (1978) Biochemistry 17:3448-3453). Purified *A. victoria* GFP is a monomeric protein of about 27 Kda that absorbs blue light with excitation wavelength maximum of 395 nm, with a minor peak at 470 nm, and emits green fluorescence with an

25 emission wavelength of about 510 nm and a minor peak near 540 nm (Ward *et al.* (1979) Photochem. Photobiol. Rev 4:1-57). This GFP has certain limitations. The excitation maximum of the wildtype GFP is not within the range of wavelengths of standard fluorescein detection optics.

The detection of green fluorescence does not require any exogenous

30 substrates or co-factors. Instead, the high level of fluorescence results from the intrinsic chromophore of the protein. The chromophore includes modified amino acid residues within the polypeptide chain. For example, the fluorescent

-6-

chromophore of *A. victoria* GFP is encoded by the hexapeptide sequence, FSYGVQ, encompassing amino acid residues 64-69. The chromophore is formed by the intramolecular cyclization of the polypeptide backbone at residues Ser65 and Gly67 and the oxidation of the  $\alpha$ - $\beta$  bond of residue Tyr66 (e.g., see  
5 Cody *et al.* (1993) Biochemistry 32:1212-1218; Shimomura (1978) FEBS Letters 104:220-222; Ward *et al.* (1989) Photochem. Photobiol. 49:62S). The emission spectrum of the isolated chromophore and the denatured protein at neutral Ph do not match the spectrum of the native protein, suggesting that chromophore formation occurs post-translationally (e.g., see Cody *et al.* (1993)  
10 Biochemistry 32:1212-1218).

In addition, the crystal structure of purified *A. victoria* GFP has been determined (e.g., see Ormö (1996) Science 273:1392-1395). The predominant structural features of the protein are an 11-stranded  $\beta$  barrel that forms a nearly perfect cylinder wrapping around a single central  $\alpha$ -helix, which contains the  
15 modified *p*-hydroxybenzylideneimidazolidinone chromophore. The chromophore is centrally located within the barrel structure and is completely shielded from exposure to bulk solvent.

DNA encoding an isotype of *A. victoria* GFP has been isolated and its nucleotide sequence has been determined (e.g., see Prasher (1992) Gene  
20 111:229-233). The *A. victoria* CDNA contains a 714 nucleotide open reading frame that encodes a 238 amino acid polypeptide of a calculated  $M_r$  of 26,888 Da. Recombinantly expressed *A. victoria* GFPs retain their ability to fluoresce *in vivo* in a wide variety organisms, including bacteria (e.g., see Chalfie *et al.* (1994) Science 263:802-805; Miller *et al.* (1997) Gene 191:149-153), yeast  
25 and fungi (Fey *et al.* (1995) Gene 165:127-130; Straight *et al.* (1996) Curr. Biol. 6:1599-1608; Cormack *et al.* (1997) Microbiology 143:303-311), *Drosophila* (e.g., see Wang *et al.* (1994) Nature 369:400-403; Plautz (1996) Gene 173:83-87), plants (Heinlein *et al.* (1995); Casper *et al.* (1996) Gene 173:69-73), fish (Amsterdam *et al.* (1995) ), and mammals (Ikawa *et al.*  
30 (1995). *Aequorea* GFP vectors and isolated *Aequorea* GFP proteins have been

-7-

used as markers for measuring gene expression, cell migration and localization, microtubule formation and assembly of functional ion channels (e.g., see Terry *et al.* (1995) Biochem. Biophys. Res. Commun. 217:21-27; Kain *et al.* (1995) Biotechniques 19:650-655). The *A. victoria* GFP, however, is not ideal for use in

5 analytical and diagnostic processes. Consequently GFP mutants have been selected with the hope of identifying mutants that have single excitation spectral peaks shifted to the red.

In fact a stated purpose in constructing such mutants has been to attempt to make the *A. victoria* GFP more like the GFP from *Renilla*, which has

10 thus far not been cloned, but which has properties that make it far more ideal for use as an analytical tool. For many practical applications, the spectrum of *Renilla* GFP would be preferable to that of the *Aequorea* GFP, because wavelength discrimination between different fluorophores and detection of resonance energy transfer are easier if the component spectra are tall and

15 narrow rather than low and broad [see, U.S. Patent No. 5,625,048]. Furthermore, the longer wavelength excitation peak (475 nm) of *Renilla* GFP is almost ideal for fluorescein filter sets and is resistant to photobleaching, but has lower amplitude than the shorter wavelength peak at 395 nm, which is more susceptible to photobleaching [Chalfie *et al.* (1994) Science 263:802-805 ].

20 There exists a phylogenetically diverse and largely unexplored repertoire of bioluminescent proteins that are a reservoir for future development. Many of these, such as nucleic acid encoding *Renilla* GFPs have not, despite concentrated efforts to do so.

For these reasons, it would be desirable to have a variety of new

25 luciferases and fluorescent proteins, particularly, *Renilla* GFP available rather than use mutants of *A. victoria* GFP. It has, not, however, been possible to clone the gene encoding any *Renilla* GFPs. It would also be desirable to have a variety of GFPs and luciferases available in order to optimize systems for particular applications and to improve upon existing methods. Therefore, it is

30 an object herein to provide isolated nucleic acids encoding heretofore unavailable luciferases and the protein encoded thereby. It is also an object herein to provide isolated nucleic acids encoding *Renilla* GFPs, GFPs from other

-8-

species, and luciferases from a variety of species, and the proteins encoded thereby. It is also an object herein to provide bioluminescence generating systems that include the luciferases, luciferins, and also include GFPs.

#### SUMMARY OF THE INVENTION

5 Isolated nucleic acids that encode fluorescent proteins and nucleic acids that encode luciferases are provided. Nucleic acid molecules encoding GFPs from *Renilla* and from *Ptilosarcus* are provided.

Nucleic acid molecules that encode the *Renilla mulleri* luciferase, a *Gaussia* species luciferase and a *Pleuromamma* species luciferase are provided. Nucleic  
10 acid probes derived therefrom are also provided. Functionally equivalent nucleic acids, such as those that hybridize under conditions of high stringency to the disclosed molecules, are also contemplated.

Host cells, including bacterial, yeast and mammalian host cells, and plasmids for expression of the nucleic acids encoding each luciferase and GFP  
15 and combinations of luciferases and GFPs are also provided in these hosts are also provided. The genes can be modified by substitution of codons optimized for expression in selected host cells or hosts, such as humans and other mammals, or can be mutagenized to alter the emission properties.

#### Luciferases

20 Recombinant host cells, including bacterial, yeast and mammalian cells, containing heterologous nucleic acid encoding a *Renilla mulleri* luciferase and the nucleic acid are provided. In preferred embodiments, the heterologous nucleic acid encodes the sequence of amino acids set forth in SEQ ID No. 18. In more preferred embodiments, the heterologous nucleic acid encodes the  
25 sequence of nucleotides set forth in SEQ ID No. 17. Also provided are functionally equivalent nucleic acids, such as nucleic acid molecules that hybridize under moderate or high stringency to the sequence of nucleotides set forth in SEQ ID No. 17, particularly when using the probes provided herein.

Isolated nucleic acids that encode luciferases from *Gaussia* are provided  
30 herein. In particular, nucleic acid fragments that encode *Gaussia princeps* luciferase, and nucleic acid probes derived therefrom are provided. In a particular embodiment, the luciferase is encoded by the sequence of nucleotides

-9-

set forth in SEQ ID No. 19. Also provided are nucleic acid molecules that hybridize under moderate or high stringency to the sequence of nucleotides set forth in SEQ ID No. 19, particularly when using probes provided herein. Probes derived from this nucleic acid that can be used in methods provided herein to  
5 isolate luciferases from any *Gaussia* species are provided. In an exemplary embodiment, nucleic acid encoding *Gaussia princeps* luciferase is provided. This nucleic acid encodes the sequence of amino acids set forth in SEQ ID No. 20.

Nucleic acids that encode *Pleuromamma* luciferase are provided. In  
10 particular, a nucleic acid molecule that encodes a *Pleuromamma* luciferase and the encoded luciferase are set forth in SEQ ID Nos. 28 and 29, respectively. Nucleic acid encoding a *Pleuromamma* luciferase has also been isolated.

Expression vectors that contain DNA encoding a *Renilla mulleri*, *Gaussia* or *Pleuromamma* luciferase linked in operational association with a promoter  
15 element that allows for the constitutive or inducible expression of the luciferase are provided. In preferred embodiments, the vectors are capable of expressing the *Renilla mulleri* luciferase in a wide variety of host cells. Vectors for producing chimeric *Renilla mulleri* luciferase fusion proteins, preferably chimeric antibody-luciferase or acetylcholine esterase fusion proteins, containing a  
20 promoter element and a multiple cloning site located upstream or downstream of DNA encoding *Renilla mulleri* luciferase are also provided.

Recombinant cells containing heterologous nucleic acid encoding a *Gaussia* luciferase are also provided. Purified *Gaussia* luciferases and compositions containing a *Gaussia* luciferase alone or in combination with at  
25 other components of a bioluminescence-generating system, such as a *Renilla* green fluorescent protein, are provided. The *Gaussia* luciferase can be used, for example, to provide fluorescent illumination of novelty items or used in methods of detecting and visualizing neoplastic tissue and other tissues, detecting infectious agents using immunoassays, such homogenous immunoassays and in  
30 vitro fluorescent-based screening assays using multi-well assay devices, or provided in kits for carrying out any of the above-described methods. In particular, the *Gaussia*

-10-

luciferase may be used in conjunction with suitable fluorescent proteins in assays provided herein.

Methods using the probes for the isolation and cloning of luciferase-encoding DNA in *Gaussia*, *Pleuromamma* and other species are also provided.

- 5 In preferred embodiments, the nucleic acid probes are degenerate probes of at least 14 nucleotides, preferably 16 to 30 nucleotides, that are based on amino acids set forth in SEQ ID No. 19 and or the sequence of nucleotides set forth in SEQ ID No. 29.

- Vectors containing DNA encoding a *Gaussia* luciferase or *Pleuromamma* luciferase are provided. In particular, expression vectors that contain DNA encoding the luciferase linked in operational association with a promoter element that allows for the constitutive or inducible expression of luciferase are provided. In preferred embodiments, the vectors are capable of expressing the luciferase in a wide variety of host cells. Vectors for producing chimeric
- 10 luciferase fusion proteins (see, e.g., U.S. Patent No. 5,464,745, which describes the use of protein binding domains; see SEQ ID Nos. 21 and 22, which set forth the sequences of a cellulose binding domain-luciferase fusion protein; and which are depicted in FIGS. 1 and 2) containing a promoter element and a multiple cloning site located upstream or downstream from DNA encoding
- 15 *Gaussia* or *Pleuromamma* luciferase are also provided. In a particular embodiment, DNA encoding the luciferase is linked to DNA encoding the N-terminal portion of the cellulose binding domain (CBD<sub>ctos</sub>; see, SEQ ID Nos. 21 and 22) in a PET vector (Novagen; see, U.S. Patent Nos. 5,719,044 and 5,738,984, 5,670,623 and 5,496,934 and the Novagen catalog; complete
- 20 sequences of each PET vector are provided with purchase of the vector).
- 25

- Fusions of the nucleic acid, particularly DNA, encoding a *Gaussia* or *Pleuromamma* luciferase with DNA encoding a GFP or phycobiliprotein are also provided herein. Also provided are fusions of *Renilla* luciferase and a *Renilla* GFP. In these fusions the luciferase and GFP encoding DNA can be contiguous
- 30 or separated by a spacer peptide. The fusions are used to produce fusion proteins, which by virtue of the interaction between the luciferase and GFP pair have a variety of unique analytical applications. The interaction is assessed by



-11-

the emission spectrum of the luciferase-GFP protein pair in the presence of a luciferin and appropriate binding factors.

Recombinant host cells containing heterologous nucleic acid encoding a *Gaussia* or *Pleuromamma* luciferase are provided. In certain embodiments, the recombinant cells that contain the heterologous DNA encoding the luciferase are produced by transfection with DNA encoding a luciferase or by introduction of RNA transcripts of DNA encoding the protein. The DNA may be introduced as a linear DNA fragment or may be included in an expression vector for stable or transient expression of the encoding DNA.

The cells that express functional luciferase may be used alone or in conjunction with a bioluminescence-generating system, in cell-based assays and screening methods, such as those described herein. Presently preferred host cells for expressing the luciferase are bacteria, yeasts, fungi, plant cells, insect cells and animal cells.

Purified *Gaussia*, *Pleuromamma* and *Renilla mulleri* luciferases are provided. These luciferases are preferably obtained by expression of the nucleic acid provided herein in prokaryotic or eukaryotic cells that contain the nucleic acid that encodes the luciferase protein; and isolation of the expressed protein.

Compositions containing the luciferases are provided. The compositions can take any of a number of forms, depending on the intended method of use therefor. In certain embodiments, for example, the compositions contain a *Gaussia* luciferase, *Gaussia* luciferase peptide or *Gaussia* luciferase fusion protein, formulated for use in luminescent novelty items, immunoassays, donors in FET [fluorescent energy transfer] assays, FRET [fluorescent resonance energy transfer] assays, HTRF [homogeneous time-resolved fluorescence] assays or used in conjunction with multi-well assay devices containing integrated photodetectors, such as those described herein.

In more preferred embodiments, the bioluminescence-generating system includes, in addition to the luciferase a *Renilla mulleri* or *Ptilosarcus* GFP. These compositions can be used in a variety of methods and systems, such as

-12-

included in conjunction with diagnostic systems for the *in vivo* detection of neoplastic tissues and other tissues, such as those methods described herein.

- Combinations containing a first composition containing a luciferase and a second composition containing one or more additional components of a bioluminescence-
- 5 generating system for use with articles of manufacture to produce novelty items are provided. These novelty items are designed for entertainment, recreation and amusement, and include, but are not limited to: toys, particularly squirt guns, toy cigarettes, toy "Halloween" eggs, footbags and board/card games; finger paints and other paints, slimy play material; textiles, particularly
- 10 clothing, such as shirts, hats and sports gear suits, threads and yarns; bubbles in bubble making toys and other toys that produce bubbles; balloons; figurines; personal items, such as cosmetics, bath powders, body lotions, gels, powders and creams, nail polishes, make-up, toothpastes and other dentifrices, soaps, body paints, and bubble bath; items such as inks, paper; foods, such as
- 15 gelatins, icings and frostings; fish food containing luciferins and transgenic fish, particularly transgenic fish that express a luciferase; plant food containing a luciferin or luciferase, preferably a luciferin for use with transgenic plants that express luciferase; and beverages, such as beer, wine, champagne, soft drinks, and ice cubes and ice in other configurations; fountains, including liquid
- 20 "fireworks" and other such jets or sprays or aerosols of compositions that are solutions, mixtures, suspensions, powders, pastes, particles or other suitable form.

- Any article of manufacture that can be combined with a bioluminescence-generating system as provided herein and thereby provide
- 25 entertainment, recreation and/or amusement, including use of the items for recreation or to attract attention, such as for advertising goods and/or services that are associated with a logo or trademark is contemplated herein. Such uses may be in addition to or in conjunction with or in place of the ordinary or normal use of such items. As a result of the combination, the items glow or produce,
- 30 such as in the case of squirt guns and fountains, a glowing fluid or spray of liquid or particles. The novelty in the novelty item derives from its bioluminescence.

-13-

### GFPS

Isolated nucleic acids that encode GFPs from *Renilla* are provided herein. Also provided are isolated and purified nucleic acids that encode a component of the bioluminescence generating system and a green fluorescent protein (GFP) of a member of the genus *Renilla*, and the proteins encoded thereby are provided. In particular, nucleic acid fragments that encode *Renilla* green fluorescent protein (GFPs) and the *Renilla mulleri* luciferase, and nucleic acid probes derived therefrom are provided.

Nucleic acid molecules encoding *Renilla* GFP are provided. In particular, nucleic acid molecules encoding a *Renilla* GFP that includes the coding portion of the sequence of nucleotides set forth in SEQ ID No. 15 or that hybridizes under moderate or high stringency to the sequence of nucleotides set forth in SEQ ID No. 15, particularly when using probes provided herein, are provided. Probes derived from this nucleic acid that can be used in methods provided herein to isolated GFPs from any *Renilla* species. In an exemplary embodiment, nucleic acid encoding *Renilla mulleri* GFP is provided. This nucleic acid encodes the sequence of amino acids set forth in SEQ ID No. 16.

Nucleic acid probes can be labeled, which if needed, for detection, containing at least about 14, preferably at least about 16, or, if desired, 20 or 30 or more, contiguous nucleotides of sequence of nucleotides encoding a *Renilla* GFP, particularly *Renilla mulleri*. In preferred embodiments, the nucleic acid probes for the *Renilla* GFP are selected from the sequence of nucleotides set forth in SEQ ID No. 15.

Methods using the probes for the isolation and cloning of GFP-encoding DNA in *Renilla* and other species are also provided. In preferred embodiments, the nucleic acid probes are degenerate probes based upon the conserved regions between the *Renilla* species of GFP as set forth in Figure 3. Such degenerate probes contain at least 14 nucleotides, preferably 16 to 30 nucleotides, that are based on amino acids 51 to 68, 82 to 98 and 198 to 208 set forth in SEQ ID No. 16, amino acid sequence set forth in SEQ ID No. 24, amino acids 9-20 set forth in SEQ ID No. 25 and amino acids 39-53 set forth in SEQ ID No. 27. In other preferred embodiments, the nucleic acid probes

-14-

encoding the above-described preferred amino acid regions are selected among the sequence of nucleotides encoding these regions as set forth in SEQ ID NO. 15. Alternatively, nucleic acids, particularly those set forth in SEQ ID No. 15 that encode the noted regions may be used as primers for PCR amplification of  
5 libraries of a selected *Renilla* species, whereby DNA comprising that encodes a *Renilla* GFP is isolated.

Nucleic acids that encode a *Ptilosarcus* GFP are set forth in SEQ ID Nos. 30 and 31; the encoded GFP is set forth in SEQ ID No. 32. Also provided are nucleic acid molecules that hybridize under moderate or high stringency to the  
10 sequence of nucleotides set forth in SEQ ID Nos. 28, 30 and 31.

Vectors containing DNA encoding a *Renilla* or *Ptilosarcus* GFP are provided. In particular, expression vectors that contain DNA encoding a *Renilla* or *Ptilosarcus* GFP linked in operational association with a promoter element that allows for the constitutive or inducible expression of *Renilla* or *Ptilosarcus*  
15 GFP are provided. Native *Renilla* GFP has been expressed.

The vectors are capable of expressing the *Renilla* GFP in a wide variety of host cells. Vectors for producing chimeric *Renilla* GFP fusion proteins containing a promoter element and a multiple cloning site located upstream or downstream of DNA encoding *Renilla* GFP are also provided.

20 Recombinant cells containing heterologous nucleic acid encoding a *Ptilosarcus* GFP, *Renilla* GFP, *Renilla mulleri* luciferase, *Gaussia* luciferase, and *Pleuromamma* luciferase are also provided. Purified *Renilla mulleri* GFP, *Renilla reniformis* GFP peptides and compositions containing a *Renilla* GFPs and GFP peptides alone or in combination with at least one component of a  
25 bioluminescence-generating system, such as a *Renilla mulleri* luciferase, are provided. The *Renilla* GFP and GFP peptide compositions can be used, for example, to provide fluorescent illumination of novelty items or used in methods of detecting and visualizing neoplastic tissue and other tissues, detecting infectious agents using immunoassays, such homogenous immunoassays and in  
30 vitro fluorescent-based screening assays using multi-well assay devices, or provided in kits for carrying out any of the above-described methods. In particular, these proteins may be used in FP [fluorescence polarization] assays,

-15-

FET [fluorescent energy transfer] assays, FRET [fluorescent resonance energy transfer] assays and HTRF [homogeneous time-resolved fluorescence] assays and also in the BRET assays and sensors provided herein.

Non-radioactive energy transfer reactions, such as FET or FRET, FP and  
5 HTRF assays, are homogeneous luminescence assays based on energy transfer are carried out between a donor luminescent label and an acceptor label [see, e.g., Cardullo *et al.* (1988) *Proc. Natl. Acad. Sci. U.S.A.* 85:8790-8794; Pearce *et al.* (1986) *Proc. Natl. Acad. Sci. U.S.A.* 83:8092-8096; U.S. Patent No. 4,777,128; U.S. Patent No. 5,162,508; U.S. Patent No. 4,927,923; U.S.  
10 Patent No. 5,279,943; and International PCT Application No. WO 92/01225]. Non-radioactive energy transfer reactions using GFPs have been developed [see, International PCT application Nos. WO 98/02571 and WO 97/28261]. Non-radioactive energy transfer reactions using GFPs and luciferases, such as a luciferase and its cognate GFP (or multimers thereof), such as in a fusion  
15 protein, are contemplated herein.

Nucleic acids that exhibit substantial sequence identity with the nucleic acids provided herein are also contemplated. These are nucleic acids that can be produced by substituting codons that encode conservative amino acids and also nucleic acids that exhibit at least about 80%, preferably 90 or 95%  
20 sequence identity. Sequence identity refers to identity as determined using standard programs with default gap penalties and other defaults as provided by the manufacturer thereof.

The nucleic acids provide an opportunity to produce luciferases and GFPs, which have advantageous application in all areas in which  
25 luciferase/luciferins and GFPs have application. The nucleic acids can be used to obtain and produce GFPs and GFPs from other, particularly *Renilla* species using the probes described herein that correspond to conserved regions (see, e.g., Figure 3). These GFPs have advantageous application in all areas in which GFPs and/or luciferase/luciferins have application. For example, The GFP's provide a  
30 means to amplify the output signal of bioluminescence generating systems. *Renilla* GFP has a single excitation absorbance peak in blue light (and around 498 nm) and a predominantly single emission peak around 510 nm (with a small

-16-

shoulder near 540). This spectra provides a means for it to absorb blue light and efficiently convert it to green light. This results in an amplification of the output. When used in conjunction with a bioluminescence generating system that yields blue light, such as *Aequorea* or *Renilla* or *Vargula* (*Cypridina*), the output signal for any application, including diagnostic applications, is amplified. In addition, this green light can serve as an energy donor in fluorescence-based assays, such as fluorescence polarization assays, FET [fluorescent energy transfer] assays, FRET [fluorescent resonance energy transfer] assays and HTRF [homogeneous time-resolved fluorescence] assays. Particular assays, herein referred to as BRET [bioluminescence resonance energy transfer assays in which energy is transferred from a bioluminescence reaction of a luciferase to a fluorescent protein], are provided.

Non-radioactive energy transfer reactions, such as FET or FRET, FP and HTRF assays, are homogeneous luminescence assays based on energy transfer that are carried out between a donor luminescent label and an acceptor label [see, e.g., Cardullo et al. (1988) Proc. Natl. Acad. Sci. U.S.A. 85:8790-8794; Pearce et al. (1986) Proc. Natl. Acad. Sci. U.S.A. 83:8092-8096; U.S. Patent No. 4,777,128; U.S. Patent No. 5,162,508; U.S. Patent No. 4,927,923; U.S. Patent No. 5,279,943; and International PCT Application No. WO 92/01225]. Non-radioactive energy transfer reactions using GFPs have been developed [see, International PCT application Nos. WO 98/02571 and WO 97/28261].

Mutagenesis of the GFPs is contemplated herein, particularly mutagenesis that results in modified GFPs that have red-shifted excitation and emission spectra. The resulting systems have higher output compared to the unmutagenized forms. These GFPs may be selected by random mutagenesis and selection for GFPs with altered spectra or by selected mutagenesis of the chromophore region of the GFP.

Recombinant host cells containing heterologous nucleic acid encoding a *Renilla* or *Ptilosarcus* GFP are also provided. In certain embodiments, the recombinant cells that contain the heterologous DNA encoding the *Renilla* or *Ptilosarcus* GFP are produced by transfection with DNA encoding a *Renilla* or *Ptilosarcus* GFP or by introduction of RNA transcripts of DNA encoding a *Renilla*

-17-

or *Ptilosarcus* protein. The DNA may be introduced as a linear DNA fragment or may be included in an expression vector for stable or transient expression of the encoding DNA.

In certain embodiments, the cells contain DNA or RNA encoding a *Renilla*  
5 *mulleri* GFP or a *Ptilosarcus* GFP (particularly from a species other than *P. gurneyi*) also express the recombinant *Renilla mulleri* GFP or *Ptilosarcus* polypeptide. It is preferred that the cells are selected to express functional GFPs that retain the ability to fluorescence and that are not toxic to the host cell. In some embodiments, cells may also include heterologous nucleic acid  
10 encoding a component of a bioluminescence-generating system, preferably a photoprotein or luciferase. In preferred embodiments, the nucleic acid encoding the bioluminescence-generating system component is isolated from the species *Aequorea*, *Vargula*, *Pleuromamma*, *Ptilosarcus* or *Renilla*. In more preferred embodiments, the bioluminescence-generating system component is a *Renilla*  
15 *mulleri* luciferase including the amino acid sequence set forth in SEQ ID No. 18 or the *Pleuromamma* luciferase set forth in SEQ ID No. 28, or the *Gaussia* luciferase set forth in SEQ ID No. 19.

The GFPs provided herein may be used in combination with any suitable bioluminescence generating system, but is preferably used in combination with  
20 a *Renilla* or *Aequorea*, *Pleuromamma* or *Gaussia* luciferase.

Purified *Renilla* GFPs, particularly *Renilla mulleri* GFP, and purified *Renilla reniformis* GFP peptides are provided. Presently preferred *Renilla* GFP for use in the compositions herein is *Renilla mulleri* GFP including the sequence of amino acids set forth in SEQ ID No. 16. Presently preferred *Renilla reniformis* GFP  
25 peptides are those containing the GFP peptides selected from the amino acid sequences set forth in SEQ ID Nos 19-23.

The *Renilla* GFP, GFP peptides and luciferase can be isolated from natural sources or isolated from a prokaryotic or eukaryotic cell transfected with nucleic acid that encodes the *Renilla* GFP and/or luciferase protein.

30 Fusions of the nucleic acid, particularly DNA, encoding *Renilla* or *Ptilosarcus* GFP with DNA encoding a luciferase are also provided herein.

-18-

The cells that express functional luciferase and/or GFP, which may be used alone or in conjunction with a bioluminescence-generating system, in cell-based assays and screening methods, such as those described herein.

Presently preferred host cells for expressing GFP and luciferase are  
5 bacteria, yeasts, fungi, plant cells, insect cells and animal cells.

The luciferases and GFPs or cells that express them also may be used in methods of screening for bacterial contamination and methods of screening for metal contaminants. To screen for bacterial contamination, bacterial cells that express the luciferase and/or GFP are put in autoclaves or in other areas in  
10 which testing is contemplated. After treatment or use of the area, the area is tested for the presence of glowing bacteria. Presence of such bacteria is indicative of a failure to eradicate other bacteria. Screening for heavy metals and other environmental contaminants can also be performed with cells that contain the nucleic acids provided herein, if expression is linked to a system  
15 that is dependent upon the particular heavy metal or contaminant.

The systems and cells provided herein can be used for high throughout screening protocols, intracellular assays, medical diagnostic assays, environmental testing, such as tracing bacteria in water supplies, in conjunction with enzymes for detecting heavy metals, in spores for testing autoclaves in  
20 hospital, foods and industrial autoclaves. Non-pathogenic bacteria containing the systems can be included in feed to animals to detect bacterial contamination in animal products and in meats.

Compositions containing a *Renilla* or *Ptilosarcus* GFP are provided. The compositions can take any of a number of forms, depending on the intended  
25 method of use therefor. In certain embodiments, for example, the compositions contain a *Renilla* GFP or GFP peptide, preferably *Renilla mulleri* GFP or *Renilla reniformis* GFP peptide, formulated for use in luminescent novelty items, immunoassays, FET [fluorescent energy transfer] assays, FRET [fluorescent resonance energy transfer] assays, HTRF [homogeneous time-resolved  
30 fluorescence] assays or used in conjunction with multi-well assay devices containing integrated photodetectors, such as those described herein. In other instances, the GFPs are used in beverages, foods or cosmetics.



-19-

- Compositions that contain a *Renilla mulleri* GFP or GFP peptide and at least one component of a bioluminescence-generating system, preferably a luciferase, luciferin or a luciferase and a luciferin, are provided. In preferred embodiments, the luciferase/luciferin bioluminescence-generating system is
- 5 selected from those isolated from: an insect system, a coelenterate system, a ctenophore system, a bacterial system, a mollusk system, a crustacea system, a fish system, an annelid system, and an earthworm system. Bioluminescence-generating systems include those isolated from *Renilla*, *Aequorea*, and *Vargula*, *Gaussia* and *Pleuromamma*.
- 10 Combinations containing a first composition containing a *Renilla mulleri* GFP or *Ptilosarcus* GFP or mixtures thereof and a second composition containing a bioluminescence-generating system for use with inanimate articles of manufacture to produce novelty items are provided. These novelty items, which are articles of manufacture, are designed for entertainment, recreation
- 15 and amusement, and include, but are not limited to: toys, particularly squirt guns, toy cigarettes, toy "Halloween" eggs, footbags and board/card games; finger paints and other paints, slimy play material; textiles, particularly clothing, such as shirts, hats and sports gear suits, threads and yarns; bubbles in bubble making toys and other toys that produce bubbles; balloons; figurines; personal
- 20 items, such as bath powders, body lotions, gels, powders and creams, nail polishes, cosmetics including make-up, toothpastes and other dentifrices, soaps, cosmetics, body paints, and bubble bath, bubbles made from non-detergent sources, particularly proteins such as albumin and other non-toxic proteins; in fishing lures, particularly crosslinked polyacrylamide containing a fluorescent
- 25 protein and/or components of a bioluminescence generating system, which glow upon contact with water; items such as inks, paper; foods, such as gelatins, icings and frostings; fish food containing luciferins and transgenic fish, particularly transgenic fish that express a luciferase; plant food containing a luciferin or luciferase, preferably a luciferin for use with transgenic plants that
- 30 express luciferase; and beverages, such as beer, wine, champagne, soft drinks, and ice cubes and ice in other configurations; fountains, including liquid "fireworks" and other such jets or sprays or aerosols of compositions that are

-20-

solutions, mixtures, suspensions, powders, pastes, particles or other suitable form.

Any article of manufacture that can be combined with a bioluminescence-generating system as provided herein and thereby provide  
5 entertainment, recreation and/or amusement, including use of the items for recreation or to attract attention, such as for advertising goods and/or services that are associated with a logo or trademark is contemplated herein. Such uses may be in addition to or in conjunction with or in place of the ordinary or normal use of such items. As a result of the combination, the items glow or produce,  
10 such as in the case of squirt guns and fountains, a glowing fluid or spray of liquid or particles.

Methods for diagnosis and visualization of tissues *in vivo* or *in situ* using compositions containing a *Renilla mulleri* GFP and/or a *Renilla mulleri* luciferase or others of the luciferases and/or GFPs provided herein are provided. For  
15 example, the *Renilla mulleri* GFP protein can be used in conjunction with diagnostic systems that rely on bioluminescence for visualizing tissues *in situ*. The systems are particularly useful for visualizing and detecting neoplastic tissue and specialty tissue, such as during non-invasive and invasive procedures. The systems include compositions containing conjugates that  
20 include a tissue specific, particularly a tumor-specific, targeting agent linked to a targeted agent, a *Renilla mulleri* GFP, a luciferase or luciferin. The systems also include a second composition that contains the remaining components of a bioluminescence generating reaction and/or the *Renilla mulleri* GFP. In some embodiments, all components, except for activators, which are provided *in situ*  
25 or are present in the body or tissue, are included in a single composition.

Methods for diagnosis and visualization of tissues *in vivo* or *in situ* using compositions containing a *Gaussia* luciferase are provided. For example, the *Gaussia* luciferase or *Gaussia* luciferase peptide can be used in conjunction with  
30 diagnostic systems that rely on bioluminescence for visualizing tissues *in situ*. The systems are particularly useful for visualizing and detecting neoplastic tissue and specialty tissue, such as during non-invasive and invasive procedures. The systems include compositions containing conjugates that

-21-

include a tissue specific, particularly a tumor-specific, targeting agent linked to a targeted agent, a *Gaussia* luciferase, a GFP or luciferin. The systems also include a second composition that contains the remaining components of a bioluminescence generating reaction and/or the *Gaussia* luciferase. In some  
5       embodiments, all components, except for activators, which are provided *in situ* or are present in the body or tissue, are included in a single composition.

In particular, the diagnostic systems include two compositions. A first composition that contains conjugates that, in preferred embodiments, include antibodies directed against tumor antigens conjugated to a component of the  
10       bioluminescence generating reaction, a luciferase or luciferin, preferably a luciferase are provided. In certain embodiments, conjugates containing tumor-specific targeting agents are linked to luciferases or luciferins. In other embodiments, tumor-specific targeting agents are linked to microcarriers that are coupled with, preferably more than one of the bioluminescence generating  
15       components, preferably more than one luciferase molecule.

The second composition contains the remaining components of a bioluminescence generating system, typically the luciferin or luciferase substrate. In some embodiments, these components, particularly the luciferin are linked to a protein, such as a serum albumin, or other protein carrier. The  
20       carrier and time release formulations, permit systemically administered components to travel to the targeted tissue without interaction with blood cell components, such as hemoglobin that deactivates the luciferin or luciferase..

Methods for diagnosing diseases, particularly infectious diseases, using chip methodology (see, e.g., copending U.S. application Serial No. 08/990,103)  
25       a luciferase/luciferin bioluminescence-generating system and a *Renilla mulleri* or *Ptilosarcus* GFP are provided. In particular, the chip includes an integrated photodetector that detects the photons emitted by the bioluminescence-generating system, particularly using luciferase encoded by the nucleic acids provided herein and/or *Renilla mulleri* or *Ptilosarcus* GFP.

30       In one embodiment, the chip is made using an integrated circuit with an array, such as an X-Y array, of photodetectors. The surface of circuit is treated to render it inert to conditions of the diagnostic assays for which the chip is

-22-

- intended, and is adapted, such as by derivatization for linking molecules, such as antibodies. A selected antibody or panel of antibodies, such as an antibody specific for a bacterial antigen, is affixed to the surface of the chip above each photodetector. After contacting the chip with a test sample, the chip is
- 5 contacted with a second antibody linked to a *Renilla* or *Pleuromamm* GFP, a chimeric antibody-*Renilla* GFP fusion protein or an antibody linked to a component of a bioluminescence generating system, such as a luciferase or luciferin, that are specific for the antigen. The remaining components of the bioluminescence generating reaction are added, and, if any of the antibodies
- 10 linked to a component of a bioluminescence generating system are present on the chip, light will be generated and detected by the adjacent photodetector. The photodetector is operatively linked to a computer, which is programmed with information identifying the linked antibodies, records the event, and thereby identifies antigens present in the test sample.
- 15 Methods for generating chimeric GFP fusion proteins are provided. The methods include linking DNA encoding a gene of interest, or portion thereof, to DNA encoding a GFP coding region in the same translational reading frame. The encoded-protein of interest may be linked in-frame to the amino- or carboxyl-terminus of the GFP. The DNA encoding the chimeric protein is then linked in
- 20 operable association with a promoter element of a suitable expression vector. Alternatively, the promoter element can be obtained directly from the targeted gene of interest and the promoter-containing fragment linked upstream of the GFP coding sequence to produce chimeric GFP proteins.
- Methods for identifying compounds using recombinant cells that express
- 25 heterologous DNA encoding a GFP under the control of a promoter element of a gene of interest are provided. The recombinant cells can be used to identify novel compounds or ligands that modulate the level of transcription from the promoter of interest by measuring GFP-mediated fluorescence. Recombinant cells expressing the chimeric *Renilla* or *Ptilosarcus* GFPs may also be used for
- 30 monitoring gene expression or protein trafficking, or determining the cellular localization of the target protein by identifying localized regions of GFP-mediated fluorescence within the recombinant cell.

-23-

Other assays using the GFPs and/or luciferases are contemplated herein. Any assay or diagnostic method known used by those of skill in the art that employ *Aequora* GFPs and/or other luciferases are contemplated herein.

Kits containing the GFPs for use in the methods, including those  
5 described herein, are provided. In one embodiment, the kits containing an article of manufacture and appropriate reagents for generating bioluminescence are provided. The kits containing such soap compositions, with preferably a moderate Ph [between 5 and 8] and bioluminescence generating reagents, including luciferase and luciferin and the GFP are provided herein. These kits,  
10 for example, can be used with a bubble-blowing or producing toy. These kits can also include a reloading or charging cartridge or can be used in connection with a food.

In another embodiment, the kits are used for detecting and visualizing neoplastic tissue and other tissues and include a first composition that contains  
15 the GFP and at least one component of a bioluminescence generating system, and a second that contains the activating composition, which contains the remaining components of the bioluminescence generating system and any necessary activating agents.

Thus, these kits will typically include two compositions, a first  
20 composition containing the GFP formulated for systemic administration (or in some embodiments local or topical application), and a second composition containing the components or remaining components of a bioluminescence generating system, formulated for systemic, topical or local administration depending upon the application. Instructions for administration will be included.

25 In other embodiments, the kits are used for detecting and identifying diseases, particularly infectious diseases, using multi-well assay devices and include a multi-well assay device containing a plurality of wells, each having an integrated photodetector, to which an antibody or panel of antibodies specific for one or more infectious agents are attached, and composition containing a  
30 secondary antibody, such as an antibody specific for the infectious agent that is linked to a *Renilla mulleri* or *Ptilosarcus* GFP protein, a chimeric antibody-*Renilla mulleri* (or *Ptilosarcus*) GFP fusion protein or F(Ab)<sub>2</sub> antibody fragment-*Renilla*

-24-

*mulleri* GFP fusion protein. A second composition containing a bioluminescence generating system that emits a wavelength of light within the excitation range of the *Renilla mulleri* GFP, such as species of *Renilla* or *Aequorea*, for exciting the *Renilla mulleri*, which produces green light that is detected by the

5 photodetector of the device to indicate the presence of the agent.

As noted above, fusions of nucleic acid encoding the luciferases and or GFPs provided herein with other luciferases and GFPs are provided. Of particular interest are fusions that encode pairs of luciferases and GFPs, such as a *Renilla* luciferase and a *Renilla* GFP (or a homodimer or other multiple of a

10 *Renilla* GFP). The luciferase and GFP bind and in the presence of a luciferin will produced fluorescence that is red shifted compared to the luciferase in the absence of the GFP. This fusion or fusions in which the GFP and luciferase are linked via a target, such as a peptide, can be used as a tool to assess anything that interacts with the linker.

15 Muteins of the GFPs and luciferases are provided. Of particular interest are muteins, such as temperature sensitive muteins, of the GFP and luciferases that alter their interaction, such as mutations in the *Renilla* luciferase and *Renilla* GFP that alters their interaction at a critical temperature.

Antibodies, polyclonal and monoclonal antibodies that specifically bind to

20 any of the proteins encoded by the nucleic acids provided herein are also provided. These antibodies, monoclonal or polyclonal, can be prepared employing standard techniques, known to those of skill in the art. In particular, immunoglobulins or antibodies obtained from the serum of an animal immunized with a substantially pure preparation of a luciferase or GFP provided herein or an

25 or epitope-containing fragment thereof are provided. Monoclonal antibodies are also provided. The immunoglobulins that are produced have, among other properties, the ability to specifically and preferentially bind to and/or cause the immunoprecipitation of a GFP or luciferase, particularly a *Renilla* or *Ptsilocarpus* GFP or a *Pleuromamma*, *Gaussia* or *Renilla mulleri* luciferase, that may be

30 present in a biological sample or a solution derived from such a biological sample.

-25-

**DESCRIPTION OF THE FIGURES**

FIGURE 1 depicts the components of the commercially available PET-34 vector (EK is enterokinase).

FIGURE 2 shows a portion of the vector with the inserted *Gaussia*-  
5 encoding luciferase.

FIGURE 3 displays an alignment of the deduced amino acid sequence of *Renilla mulleri* green fluorescent protein and the amino acid sequence of isolated *Renilla reniformis* GFP peptides obtained by proteolytic digestion of purified *Renilla reniformis* GFP. Positions in the amino acid sequence of direct identity  
10 are marked by the solid vertical lines (|) between the two *Renilla* species.

FIGURE 4 shows the fluorescence emission and excitation spectrum for the *Renilla mulleri* GFP, with a peak emission at 506 nm..

FIGURE 5 shows the fluorescence emission and excitation spectrum for the *Ptilosarcus* GFP, with a peak emission at 508.

15 FIGURE 6 shows a photoemission as a function of salt and Ph for *Pleuromamma* luciferase.

FIGURE 7 depicts the components of a *Renilla mulleri* luciferase-GFP fusion construct in pET-34; rbs: ribosome binding sequence; CDS: coding domain sequence; CBD: the cellulose binding domain; thrombin: thrombin  
20 cleavage site; EK: enterokinase cleavage site; S Tag: the RNase-S-peptide tag; and LIC: ligation independent cloning site.

FIGURE 8 shows a photoemission as a function of salt and pH for a *Gaussia* luciferase.

FIGURE 9 shows a photoemission spectrum for a *Gaussia* luciferase.

25 FIGURE 10 shows a photoemission spectrum for a *Pleuromamma* luciferase.

FIGURE 11 illustrates the underlying principle of Bioluminescent Resonance Energy Transfer (BRET) and its use as sensor: A) in isolation, a luciferase, preferably an anthozoan luciferase, emits blue light from the  
30 coelenterazine-derived chromophore; B) in isolation, a GFP, preferably an anthozoan GFP that binds to the luciferase, that is excited with blue-green light emits green light from its integral peptide based flurophore; C) when the

-26-

luciferase and GFP associate as a complex *in vivo* or *in vitro*, the luciferase non-radiatively transfers its reaction energy to the GFP fluorophore, which then emits the green light; D) any molecular interaction that disrupts the luciferase-GFP complex can be quantitatively monitored by observing the spectral shift from

5 green to blue light.

## DETAILED DESCRIPTION OF THE INVENTION

### TABLE OF CONTENTS

#### A. DEFINITIONS

#### B. BIOLUMINESCENT GENERATING SYSTEMS

10	1. Exemplary bioluminescence generating systems
	a. General description
	(1) Luciferases
	(2) Luciferins
	(3) Activators
15	(4) Reactions
	b. Ctenophore systems
	(1) The aequorin system
	(a) Aequorin and related photoproteins
	(b) Luciferin
20	(2) The <i>Renilla</i> system
	c. Crustacean, particularly <i>Cypridina</i> systems
	(1) <i>Vargula</i> luciferase
	(a) Purification from <i>Cypridina</i>
	(b) Preparation by Recombinant Methods
25	(2) <i>Vargula</i> luciferin
	(3) Reaction
	d. Insect bioluminescent systems including fireflies, click beetles, and other insect system
	(1) Luciferase
30	(2) Luciferin
	(3) Reaction
	e. Bacterial systems
	(1) Luciferases
	(2) Luciferins



-27-

- (3) Reactions
  - f. Other systems
    - (1) Dinoflagellate bioluminescence generating systems
    - (2) Systems from molluscs, such as *Latia* and *Pholas*
    - 5 (3) Earthworms and other annelids
    - (4) Glow worms
    - (5) Marine polychaete worm systems
    - (6) South American railway beetle
    - (7) Fish
  - 10 g. Fluorescent proteins
    - (1) Green and blue fluorescent proteins
    - (2) Phycobiliproteins
- C. ISOLATION AND IDENTIFICATION OF NUCLEIC ACIDS ENCODING LUCIFERASES
- 15 ISOLATION AND IDENTIFICATION OF NUCLEIC ACID ENCODING *Gaussia* LUCIFERASE
  - 1. Isolation of specimens of the genus *Gaussia*
  - 2. Preparation of *Gaussia* cDNA expression libraries
  - 20 3. Isolation and identification of DNA encoding *Gaussia* luciferase
- ISOLATION AND IDENTIFICATION OF NUCLEIC ACID ENCODING *Renilla* PROTEINS
  - 1. Isolation of specimens of the genus *Renilla*
  - 2. Preparation of *Renilla* cDNA expression libraries
  - 25 a. RNA isolation and cDNA synthesis
  - b. Construction of cDNA expression libraries
  - 30 3. Isolation and identification of DNA encoding *Renilla* GFP
  - 4. Isolation and identification of DNA encoding *Renilla* luciferase
- D. NUCLEIC ACID PROBES AND METHODS FOR ISOLATING AND CLONING LUCIFERASE- and GFP-ENCODING NUCLEIC ACIDS FROM OTHER SPECIES
- Gaussia*
- 35 NUCLEIC ACID PROBES AND METHODS FOR ISOLATING AND CLONING OF GFP-ENCODING NUCLEIC ACIDS FROM OTHER SPECIES OF *Renilla*
- Other species
- E. RECOMBINANT EXPRESSION OF PROTEINS

-28-

*Gaussia*

1. DNA encoding *Gaussia* proteins
2. DNA constructs for recombinant production of *Gaussia* proteins
3. Host organisms for recombinant production of *Gaussia* proteins
4. Methods for recombinant production of *Gaussia* proteins

*Renilla*

1. DNA encoding *Renilla* proteins
2. DNA constructs for recombinant production of *Renilla* proteins
3. Host organisms for recombinant production of *Renilla* proteins
4. Methods for recombinant production of *Renilla* proteins

## F. RECOMBINANT CELLS EXPRESSING HETEROLOGOUS NUCLEIC ACID ENCODING A LUCIFERASES AND GFPs

*Gaussia*15 RECOMBINANT CELLS EXPRESSING HETEROLOGOUS NUCLEIC ACID ENCODING A *Renilla* GREEN FLUORESCENT PROTEIN AND/OR LUCIFERASE

## G. Luciferases

H. *Renilla* and *Ptilosarcus* GFPs

## I. COMPOSITIONS

1. *Gaussia* luciferase compositions
2. *Renilla* luciferase compositions
3. *Renilla* GFP compositions
4. Conjugates
  - a. Linkers
  - b. Targeting Agents
    - 25 Anti-tumor Antigen Antibodies
    - Preparation of the conjugates
5. Formulation of the compositions for use in the
  - a. The first composition: formulation of the conjugates diagnostic systems
  - b. The second composition
  - c. Practice of the reactions in combination with targeting agents

## J. COMBINATIONS

## K. METHODS OF USE

1. Methods for diagnosis of neoplasms and other tissues
2. Methods of diagnosing diseases

-29-

3. Methods for generating chimeric *Renilla* or *Ptilosarcus* GFP, *Renilla mulleri* luciferase, *Pleuromamma* luciferase and *Gaussia* luciferase fusion proteins

4. Cell-based assays for identifying compounds

5 L. KITS

1. Dispensing and Packaging Apparatus for Combination with the GFP and Bioluminescent System Components

2. Capsules, pellets, liposomes, endosomes, vacuoles, micronized particles

10

a. Encapsulating vehicles in general

b. Encapsulating vehicles -liposomes

c. Encapsulating vehicles -gelatin and polymeric vehicles

d. Endosomes and vacuoles

e. Micronized particles

3. Immobilized systems

15

a. Matrix materials

b. Immobilization and activation

M. Bioluminescence Resonance Energy Transfer (BRET) System

N. EXAMPLES

A. DEFINITIONS

20

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. All patents and publications of referred to herein are incorporated by reference in their entirety.

25 As used herein, chemiluminescence refers to a chemical reaction in which energy is specifically channeled to a molecule causing it to become electronically excited and subsequently to release a photon thereby emitting visible light. Temperature does not contribute to this channeled energy. Thus, chemiluminescence involves the direct conversion of chemical energy to light energy.

30

As used herein, luminescence refers to the detectable EM radiation, generally, UV, IR or visible EM radiation that is produced when the excited product of an exergic chemical process reverts to its ground state with the emission of light. Chemiluminescence is luminescence that results from a chemical reaction. Bioluminescence is chemiluminescence that results from a

-30-

chemical reaction using biological molecules [or synthetic versions or analogs thereof] as substrates and/or enzymes.

As used herein, bioluminescence, which is a type of chemiluminescence, refers to the emission of light by biological molecules, particularly proteins. The  
5 essential condition for bioluminescence is molecular oxygen, either bound or free in the presence of an oxygenase, a luciferase, which acts on a substrate, a luciferin. Bioluminescence is generated by an enzyme or other protein [luciferase] that is an oxygenase that acts on a substrate luciferin [a bioluminescence substrate] in the presence of molecular oxygen and transforms  
10 the substrate to an excited state, which upon return to a lower energy level releases the energy in the form of light.

As used herein, the substrates and enzymes for producing bioluminescence are generically referred to as luciferin and luciferase, respectively. When reference is made to a particular species thereof, for clarity,  
15 each generic term is used with the name of the organism from which it derives, for example, bacterial luciferin or firefly luciferase.

As used herein, luciferase refers to oxygenases that catalyze a light emitting reaction. For instance, bacterial luciferases catalyze the oxidation of flavin mononucleotide [FMN] and aliphatic aldehydes, which reaction produces  
20 light. Another class of luciferases, found among marine arthropods, catalyzes the oxidation of *Cypridina* [*Vargula*] luciferin, and another class of luciferases catalyzes the oxidation of *Coleoptera* luciferin.

Thus, luciferase refers to an enzyme or photoprotein that catalyzes a bioluminescent reaction [a reaction that produces bioluminescence]. The  
25 luciferases, such as firefly and *Gaussia* and *Renilla* luciferases, that are enzymes which act catalytically and are unchanged during the bioluminescence generating reaction. The luciferase photoproteins, such as the aequorin photoprotein to which luciferin is non-covalently bound, are changed, such as by release of the luciferin, during bioluminescence generating reaction. The  
30 luciferase is a protein that occurs naturally in an organism or a variant or mutant thereof, such as a variant produced by mutagenesis that has one or more properties, such as thermal stability, that differ from the naturally-occurring

-31-

protein. Luciferases and modified mutant or variant forms thereof are well known. For purposes herein, reference to luciferase refers to either the photoproteins or luciferases.

Thus, reference, for example, to "*Gaussia* luciferase" means an enzyme  
 5 isolated from member of the genus *Gaussia* or an equivalent molecule obtained from any other source, such as from another related copepod, or that has been prepared synthetically. It is intended to encompass *Gaussia* luciferases with conservative amino acid substitutions that do not substantially alter activity. Suitable conservative substitutions of amino acids  
 10 are known to those of skill in this art and may be made generally without altering the biological activity of the resulting molecule. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, e.g., Watson et al. *Molecular Biology of the Gene*, 4th Edition, 1987, The  
 15 Bejacmin/Cummings Pub. co., p.224).

"*Renilla* GFP" refers to GFPs from the genus *Renilla* and to mutants or variants thereof. It is intended to encompass *Renilla* GFPs with conservative amino acid substitutions that do not substantially alter activity.

Such substitutions are preferably made in accordance with those set  
 20 forth in TABLE 1 as follows:

TABLE 1

	Original residue	Conservative substitution
	Ala (A)	Gly; Ser
	Arg (R)	Lys
25	Asn (N)	Gln; His
	Cys (C)	Ser
	Gln (Q)	Asn
	Glu (E)	Asp
	Gly (G)	Ala; Pro
30	His (H)	Asn; Gln
	Ile (I)	Leu; Val
	Leu (L)	Ile; Val
	Lys (K)	Arg; Gln; Glu
	Met (M)	Leu; Tyr; Ile
35	Phe (F)	Met; Leu; Tyr
	Ser (S)	Thr
	Thr (T)	Ser

-32-

**Original residue**

Trp (W)

Tyr (Y)

Val (V)

**Conservative substitution**

Tyr

Trp; Phe

Ile; Leu

Other substitutions are also permissible and may be determined empirically or in  
 5 accord with known conservative substitutions.

The luciferases and luciferin and activators thereof are referred to as  
 bioluminescence generating reagents or components. Typically, a subset of  
 these reagents will be provided or combined with an article of manufacture.  
 Bioluminescence will be produced upon contacting the combination with the  
 10 remaining reagents. Thus, as used herein, the component luciferases, luciferins,  
 and other factors, such as  $O_2$ ,  $Mg^{2+}$ ,  $Ca^{2+}$  are also referred to as  
 bioluminescence generating reagents [or agents or components].

As used herein, bioluminescence substrate refers to the compound that  
 is oxidized in the presence of a luciferase, and any necessary activators, and  
 15 generates light. These substrates are referred to as luciferins herein, are  
 substrates that undergo oxidation in a bioluminescence reaction. These  
 bioluminescence substrates include any luciferin or analog thereof or any  
 synthetic compound with which a luciferase interacts to generate light.  
 Preferred substrates are those that are oxidized in the presence of a luciferase  
 20 or protein in a light-generating reaction. Bioluminescence substrates, thus,  
 include those compounds that those of skill in the art recognize as luciferins.  
 Luciferins, for example, include firefly luciferin, *Cypridina* [also known as  
*Vargula*] luciferin [coelenterazine], bacterial luciferin, as well as synthetic  
 analogs of these substrates or other compounds that are oxidized in the  
 25 presence of a luciferase in a reaction the produces bioluminescence.

As used herein, capable of conversion into a bioluminescence substrate  
 means susceptible to chemical reaction, such as oxidation or reduction, that  
 yields a bioluminescence substrate. For example, the luminescence producing  
 reaction of bioluminescent bacteria involves the reduction of a flavin  
 30 mononucleotide group (FMN) to reduced flavin mononucleotide ( $FMNH_2$ ) by a  
 flavin reductase enzyme. The reduced flavin mononucleotide [substrate] then  
 reacts with oxygen [an activator] and bacterial luciferase to form an

-33-

intermediate peroxy flavin that undergoes further reaction, in the presence of a long-chain aldehyde, to generate light. With respect to this reaction, the reduced flavin and the long chain aldehyde are substrates.

As used herein, a bioluminescence generating system refers to the set of reagents required to conduct a bioluminescent reaction. Thus, the specific luciferase, luciferin and other substrates, solvents and other reagents that may be required to complete a bioluminescent reaction form a bioluminescence system. Thus a bioluminescence generating system refers to any set of reagents that, under appropriate reaction conditions, yield bioluminescence.

Appropriate reaction conditions refers to the conditions necessary for a bioluminescence reaction to occur, such as pH, salt concentrations and temperature. In general, bioluminescence systems include a bioluminescence substrate, luciferin, a luciferase, which includes enzymes luciferases and photoproteins, and one or more activators. A specific bioluminescence system may be identified by reference to the specific organism from which the luciferase derives; for example, the *Vargula* [also called *Cypridina*] bioluminescence system (or *Vargula* system) includes a *Vargula* luciferase, such as a luciferase isolated from the ostracod, *Vargula* or produced using recombinant means or modifications of these luciferases. This system would also include the particular activators necessary to complete the bioluminescence reaction, such as oxygen and a substrate with which the luciferase reacts in the presence of the oxygen to produce light.

The luciferases provided herein may be incorporated into bioluminescence generating systems and used, as appropriate, with the GFPs provided herein or with other GFPs. Similarly, the GFPs provided herein may be used with known bioluminescence generating systems.

As used herein, the amino acids, which occur in the various amino acid sequences appearing herein, are identified according to their well-known, three-letter or one-letter abbreviations. The nucleotides, which occur in the various DNA fragments, are designated with the standard single-letter designations used routinely in the art.

-34-

As used herein, a fluorescent protein refers to a protein that possesses the ability to fluoresce (i.e., to absorb energy at one wavelength and emit it at another wavelength). These proteins can be used as a fluorescent label or marker and in any applications in which such labels would be used, such as

5 immunoassays, CRET, FRET, and FET assays, and in the assays designated herein as BRET assays. For example, a green fluorescent protein refers to a polypeptide that has a peak in the emission spectrum at about 510 nm.

As used herein, the term BRET (Bioluminescence Resonance Energy Transfer) refers to non-radiative luciferase-to-FP energy transfer. It differs from

10 (Fluorescence Resonance Energy Transfer), which historically has been used for energy transfer between chemical fluors, but more recently has been applied to energy transfer between *Aequorea* GFP spectral variants.

As used herein, a BRET system refers the combination of a FP and luciferase for resonance energy transfer and BRET refers to any method

15 in which the luciferase is used to generate the light upon reaction with a luciferin which is then non-radiatively transferred to a FP. The energy is transferred to a FP, particularly a GFP, which focuses and shifts the energy and emits it at a different wavelength. In preferred embodiments, the BRET system includes a bioluminescence generating system and a GFP from the same source

20 as the luciferase in the system. A preferred pair is a *Renilla* luciferase and a *Renilla* GFP, which specifically interact. Alterations in the binding will be reflected in changes in the emission spectra of light produced by the luciferase. As a result the pair can function as a sensor of external events.

As used herein, a biosensor (or sensor) refers to a BRET system for use

25 to detect alterations in the environment *in vitro* or *in vivo* in which the BRET system is used.

As used herein, "not strictly catalytically" means that the photoprotein acts as a catalyst to promote the oxidation of the substrate, but it is changed in the reaction, since the bound substrate is oxidized and bound molecular oxygen

30 is used in the reaction. Such photoproteins are regenerated by addition of the substrate and molecular oxygen under appropriate conditions known to those of skill in this art.



-35-

As used herein, a nucleic acid probe is single-stranded DNA or RNA that has a sequence of nucleotides that includes at least 14 contiguous bases, preferably at least 16 contiguous bases, typically about 30, that are the same as (or the complement of) any 14 or more contiguous bases set forth in any of  
5 SEQ ID No., particularly SEQ ID Nos 15, 19, 21, 28, 30, 31 and also nucleic acid that encodes any of the peptides in SEQ ID Nos. 23-27. Among the preferred regions from which to construct probes include 5' and/or 3' coding sequences, sequences predicted to encode regions that are conserved among *Renilla* species. Probes from regions conserved among *Renilla* species GFPs  
10 are for isolating GFP-encoding nucleic acid from *Renilla* libraries.

In preferred embodiments, the nucleic acid probes are degenerate probes of at least 14 nucleotides, preferably 16 to 30 nucleotides, that are based on amino acids 51 to 68, 82 to 98 and 198 to 208 set forth in SEQ ID No. 16, amino acid sequence set forth in SEQ ID No. 24, amino acids 9-20 set forth in  
15 SEQ ID No. 25 and amino acids 39-53 set forth in SEQ ID No. 27. In other preferred embodiments, the nucleic acid probes encoding the above-described preferred amino acid regions are selected among the sequence of nucleotides encoding these regions set forth in SEQ ID NO. 15.

In preferred embodiments, the nucleic acid probes are degenerate probes  
20 of at least 14 nucleotides, preferably 16 to 30 nucleotides, that are based on amino acids set forth in SEQ ID No. 20. In other preferred embodiments, the nucleic acid probes encoding the above-described preferred amino acid regions are selected among the sequence of nucleotides encoding these regions set forth in SEQ ID NO. 19.

25 As used herein, vector (or plasmid) refers to discrete elements that are used to introduce heterologous DNA into cells for either expression or replication thereof. Selection and use of such vehicles are well within the skill of the artisan. An expression vector includes vectors capable of expressing DNAs that are operatively linked with regulatory sequences, such as promoter  
30 regions, that are capable of effecting expression of such DNA fragments. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into

-36-

an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome. Presently  
5 preferred plasmids for expression of *Gaussia* luciferase, *Renilla* GFP and luciferase are those that are expressed in bacteria and yeast, such as those described herein.

As used herein, a promoter region or promoter element refers to a segment of DNA or RNA that controls transcription of the DNA or RNA to which  
10 it is operatively linked. The promoter region includes specific sequences that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the promoter. In addition, the promoter region includes sequences that modulate this recognition, binding and transcription initiation activity of RNA polymerase. These  
15 sequences may be *cis* acting or may be responsive to *trans* acting factors. Promoters, depending upon the nature of the regulation, may be constitutive or regulated. Exemplary promoters contemplated for use in prokaryotes include the bacteriophage T7 and T3 promoters, and the like.

As used herein, operatively linked or operationally associated refers to  
20 the functional relationship of DNA with regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. For example, operative linkage of DNA to a promoter refers to the physical and functional relationship between the DNA and the promoter such that the transcription of such DNA is initiated from the  
25 promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA. In order to optimize expression and/or *in vitro* transcription, it may be necessary to remove, add or alter 5' untranslated portions of the clones to eliminate extra, potential inappropriate alternative translation initiation (i.e., start) codons or other sequences that may interfere  
30 with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites (see, e.g., Kozak (1991) *J. Biol. Chem.* 266:19867-19870) can be inserted immediately 5' of the start codon

-37-

and may enhance expression. The desirability of (or need for) such modification may be empirically determined.

As used herein, to target a targeted agent, such as a luciferase, means to direct it to a cell that expresses a selected receptor or other cell surface protein by linking the agent to a such agent. Upon binding to or interaction  
5 with the receptor or cell surface protein the targeted agent, can be reacted with an appropriate substrate and activating agents, whereby bioluminescent light is produced and the tumorous tissue or cells distinguished from non-tumorous tissue.

10 As used herein, an effective amount of a compound for treating a particular disease is an amount that is sufficient to ameliorate, or in some manner reduce the symptoms associated with the disease. Such amount may be administered as a single dosage or may be administered according to a regimen, whereby it is effective. The amount may cure the disease but,  
15 typically, is administered in order to ameliorate the symptoms of the disease. Repeated administration may be required to achieve the desired amelioration of symptoms.

As used herein, an effective amount of a conjugate for diagnosing a disease is an amount that will result in a detectable tissue. The tissues are  
20 detected by visualization either without aid from a detector more sensitive than the human eye, or with the use of a light source to excite any fluorescent products.

As used herein, visualizable means detectable by eye, particularly during surgery under normal surgical conditions, or, if necessary, slightly dimmed light.

25 As used herein, pharmaceutically acceptable salts, esters or other derivatives of the conjugates include any salts, esters or derivatives that may be readily prepared by those of skill in this art using known methods for such derivatization and that produce compounds that may be administered to animals or humans without substantial toxic effects and that either are pharmaceutically  
30 active or are prodrugs.

As used herein, treatment means any manner in which the symptoms of a conditions, disorder or disease are ameliorated or otherwise beneficially

-38-

altered. Treatment also encompasses any pharmaceutical use of the compositions herein.

As used herein, amelioration of the symptoms of a particular disorder by administration of a particular pharmaceutical composition refers to any  
5 lessening, whether permanent or temporary, lasting or transient that can be attributed to or associated with administration of the composition.

As used herein, substantially pure means sufficiently homogeneous to appear free of readily detectable impurities as determined by standard methods of analysis, such as thin layer chromatography (TLC), gel electrophoresis and  
10 high performance liquid chromatography (HPLC), used by those of skill in the art to assess such purity, or sufficiently pure such that further purification would not detectably alter the physical and chemical properties, such as enzymatic and biological activities, of the substance. Methods for purification of the compounds to produce substantially chemically pure compounds are known to  
15 those of skill in the art. A substantially chemically pure compound may, however, be a mixture of stereoisomers or isomers. In such instances, further purification might increase the specific activity of the compound.

As used herein, a prodrug is a compound that, upon in vivo administration, is metabolized or otherwise converted to the biologically,  
20 pharmaceutically or therapeutically active form of the compound. To produce a prodrug, the pharmaceutically active compound is modified such that the active compound will be regenerated by metabolic processes. The prodrug may be designed to alter the metabolic stability or the transport characteristics of a drug, to mask side effects or toxicity, to improve the flavor of a drug or to alter  
25 other characteristics or properties of a drug. By virtue of knowledge of pharmacodynamic processes and drug metabolism in vivo, those of skill in this art, once a pharmaceutically active compound is known, can design prodrugs of the compound (see, e.g., Nogrady (1985) Medicinal Chemistry A Biochemical Approach, Oxford University Press, New York, pages 388-392).

30 As used herein, biological activity refers to the in vivo activities of a compound or physiological responses that result upon in vivo administration of a compound, composition or other mixture. Biological activity, thus,

-39-

encompasses therapeutic effects and pharmaceutical activity of such compounds, compositions and mixtures. Biological activities may be observed in in vitro systems designed to test or use such activities. Thus, for purposes herein the biological activity of a luciferase is its oxygenase activity whereby,  
5 upon oxidation of a substrate, light is produced.

As used herein, targeting agent refers to an agent that specifically or preferentially targets a linked targeted agent, a luciferin or luciferase, to a neoplastic cell or tissue.

As used herein, tumor antigen refers to a cell surface protein expressed  
10 or located on the surface of tumor cells.

As used herein, neoplastic cells include any type of transformed or altered cell that exhibits characteristics typical of transformed cells, such as a lack of contact inhibition and the acquisition of tumor-specific antigens. Such cells include, but are not limited to leukemic cells and cells derived from a  
15 tumor.

As used herein, neoplastic disease is any disease in which neoplastic cells are present in the individual afflicted with the disease. Such diseases include, any disease characterized as cancer.

As used herein, metastatic tumors refers to tumors that are not localized  
20 in one site.

As used herein, specialty tissue refers to non-tumorous tissue for which information regarding location is desired. Such tissues include, for example, endometriotic tissue, ectopic pregnancies, tissues associated with certain disorders and myopathies or pathologies.

As used herein, a receptor refers to a molecule that has an affinity for a given ligand. Receptors may be naturally-occurring or synthetic molecules. Receptors may also be referred to in the art as anti-ligands. As used herein, the receptor and anti-ligand are interchangeable. Receptors can be used in their unaltered state or as aggregates with other species. Receptors may be  
25 attached, covalently or noncovalently, or in physical contact with, to a binding  
30 member, either directly or indirectly via a specific binding substance or linker. Examples of receptors, include, but are not limited to: antibodies, cell

-40-

membrane receptors surface receptors and internalizing receptors, monoclonal antibodies and antisera reactive with specific antigenic determinants [such as on viruses, cells, or other materials], drugs, polynucleotides, nucleic acids, peptides, cofactors, lectins, sugars, polysaccharides, cells, cellular membranes, and organelles.

Examples of receptors and applications using such receptors, include but are not restricted to:

- a) enzymes: specific transport proteins or enzymes essential to survival of microorganisms, which could serve as targets for antibiotic [ligand] selection;
- 10      b) antibodies: identification of a ligand-binding site on the antibody molecule that combines with the epitope of an antigen of interest may be investigated; determination of a sequence that mimics an antigenic epitope may lead to the development of vaccines of which the immunogen is based on one or more of such sequences or lead to the development of related diagnostic
- 15      agents or compounds useful in therapeutic treatments such as for auto-immune diseases
- c) nucleic acids: identification of ligand, such as protein or RNA, binding sites;
- d) catalytic polypeptides: polymers, preferably polypeptides, that are
- 20      capable of promoting a chemical reaction involving the conversion of one or more reactants to one or more products; such polypeptides generally include a binding site specific for at least one reactant or reaction intermediate and an active functionality proximate to the binding site, in which the functionality is capable of chemically modifying the bound reactant [see, e.g., U.S. Patent No.
- 25      5,215,899];
- e) hormone receptors: determination of the ligands that bind with high affinity to a receptor is useful in the development of hormone replacement therapies; for example, identification of ligands that bind to such receptors may lead to the development of drugs to control blood pressure; and
- 30      f) opiate receptors: determination of ligands that bind to the opiate receptors in the brain is useful in the development of less-addictive replacements for morphine and related drugs.

-41-

As used herein, antibody includes antibody fragments, such as Fab fragments, which are composed of a light chain and the variable region of a heavy chain.

As used herein, an antibody conjugate refers to a conjugate in which the  
5 targeting agent is an antibody.

As used herein, antibody activation refers to the process whereby activated antibodies are produced. Antibodies are activated upon reaction with a linker, such as heterobifunctional reagent.

As used herein, a surgical viewing refers to any procedure in which an  
10 opening is made in the body of an animal. Such procedures include traditional surgeries and diagnostic procedures, such as laparoscopies and arthroscopic procedures.

As used herein, humanized antibodies refer to antibodies that are modified to include "human" sequences of amino acids so that administration to  
15 a human will not provoke an immune response. Methods for preparation of such antibodies are known. For example, the hybridoma that expresses the monoclonal antibody is altered by recombinant DNA techniques to express an antibody in which the amino acid composition of the non-variable regions is based on human antibodies. Computer programs have been designed to identify  
20 such regions.

As used herein, ATP, AMP, NAD<sup>+</sup> and NADH refer to adenosine triphosphate, adenosine monophosphate, nicotinamide adenine dinucleotide (oxidized form) and nicotinamide adenine dinucleotide (reduced form), respectively.

As used herein, production by recombinant means by using recombinant  
25 DNA methods means the use of the well known methods of molecular biology for expressing proteins encoded by cloned DNA.

As used herein, substantially identical to a product means sufficiently similar so that the property of interest is sufficiently unchanged so that the  
30 substantially identical product can be used in place of the product.

As used herein equivalent, when referring to two sequences of nucleic acids means that the two sequences in question encode the same sequence of

-42-

amino acids or equivalent proteins. When "equivalent" is used in referring to two proteins or peptides, it means that the two proteins or peptides have substantially the same amino acid sequence with only conservative amino acid substitutions [see, e.g., Table 1, above] that do not substantially alter the activity or function of the protein or peptide. When "equivalent" refers to a property, the property does not need to be present to the same extent [e.g., two peptides can exhibit different rates of the same type of enzymatic activity], but the activities are preferably substantially the same. "Complementary," when referring to two nucleotide sequences, means that the two sequences of nucleotides are capable of hybridizing, preferably with less than 25%, more preferably with less than 15%, even more preferably with less than 5%, most preferably with no mismatches between opposed nucleotides. Preferably the two molecules will hybridize under conditions of high stringency.

As used herein: stringency of hybridization in determining percentage mismatch is as follows:

- 1) high stringency: 0.1 x SSPE, 0.1% SDS, 65°C
- 2) medium stringency: 0.2 x SSPE, 0.1% SDS, 50°C
- 3) low stringency: 1.0 x SSPE, 0.1% SDS, 50°C

It is understood that equivalent stringencies may be achieved using alternative buffers, salts and temperatures.

The term "substantially" identical or homologous or similar varies with the context as understood by those skilled in the relevant art and generally means at least 70%, preferably means at least 80%, more preferably at least 90%, and most preferably at least 95% identity.

As used herein, a composition refers to a any mixture. It may be a solution, a suspension, liquid, powder, a paste, aqueous, non-aqueous or any combination thereof.

As used herein, a combination refers to any association between two or among more items.

As used herein, fluid refers to any composition that can flow. Fluids thus encompass compositions that are in the form of semi-solids, pastes, solutions, aqueous mixtures, gels, lotions, creams and other such compositions.



-43-

Examples of receptors and applications using such receptors, include but are not restricted to:

- a) enzymes: specific transport proteins or enzymes essential to survival of microorganisms, which could serve as targets for antibiotic [ligand] selection;
- 5       b) antibodies: identification of a ligand-binding site on the antibody molecule that combines with the epitope of an antigen of interest may be investigated; determination of a sequence that mimics an antigenic epitope may lead to the development of vaccines of which the immunogen is based on one or more of such sequences or lead to the development of related diagnostic  
10       agents or compounds useful in therapeutic treatments such as for auto-immune diseases
- c) nucleic acids: identification of ligand, such as protein or RNA, binding sites;
- d) catalytic polypeptides: polymers, preferably polypeptides, that are  
15       capable of promoting a chemical reaction involving the conversion of one or more reactants to one or more products; such polypeptides generally include a binding site specific for at least one reactant or reaction intermediate and an active functionality proximate to the binding site, in which the functionality is capable of chemically modifying the bound reactant [see, e.g., U.S. Patent No.  
20       5,215,899];
- e) hormone receptors: determination of the ligands that bind with high affinity to a receptor is useful in the development of hormone replacement therapies; for example, identification of ligands that bind to such receptors may lead to the development of drugs to control blood pressure; and
- 25       f) opiate receptors: determination of ligands that bind to the opiate receptors in the brain is useful in the development of less-addictive replacements for morphine and related drugs.

As used herein, complementary refers to the topological compatibility or matching together of interacting surfaces of a ligand molecule and its receptor.  
30       Thus, the receptor and its ligand can be described as complementary, and furthermore, the contact surface characteristics are complementary to each other.

-44-

As used herein, a ligand-receptor pair or complex formed when two macromolecules have combined through molecular recognition to form a complex.

As used herein, a substrate refers to any matrix that is used either  
5 directly or following suitable derivatization, as a solid support for chemical synthesis, assays and other such processes. Preferred substrates herein, are silicon substrates or siliconized substrates that are derivitized on the surface intended for linkage of anti-ligands and ligands and other macromolecules, including the fluorescent proteins, phycobiliproteins and other emission shifters.

10 As used herein, a matrix refers to any solid or semisolid or insoluble support on which the molecule of interest, typically a biological molecule, macromolecule, organic molecule or biospecific ligand is linked or contacted. Typically a matrix is a substrate material having a rigid or semi-rigid surface. In many embodiments, at least one surface of the substrate will be substantially  
15 flat, although in some embodiments it may be desirable to physically separate synthesis regions for different polymers with, for example, wells, raised regions, etched trenches, or other such topology. Matrix materials include any materials that are used as affinity matrices or supports for chemical and biological molecule syntheses and analyses, such as, but are not limited to: polystyrene,  
20 polycarbonate, polypropylene, nylon, glass, dextran, chitin, sand, pumice, polytetrafluoroethylene, agarose, polysaccharides, dendrimers, buckyballs, polyacrylamide, Kieselguhr-polyacrylamide non-covalent composite, polystyrene-polyacrylamide covalent composite, polystyrene-PEG [polyethyleneglycol] composite, silicon, rubber, and other materials used as supports for solid phase  
25 syntheses, affinity separations and purifications, hybridization reactions, immunoassays and other such applications.

As used herein, the attachment layer refers the surface of the chip device to which molecules are linked. Typically, the chip is a semiconductor device, which is coated on a least a portion of the surface to render it suitable  
30 for linking molecules and inert to any reactions to which the device is exposed. Molecules are linked either directly or indirectly to the surface, linkage may be effected by absorption or adsorption, through covalent bonds, ionic interactions

-45-

or any other interaction. Where necessary the attachment layer is adapted, such as by derivatization for linking the molecules.

## **B. BIOLUMINESCENCE GENERATING SYSTEMS AND COMPONENTS**

5 The following is a description of bioluminescence generating systems and the components thereof. These luciferases and luciferins and fluorescent proteins can be used with the luciferases and GFPs provided herein.

### **1. Exemplary bioluminescence generating systems**

A bioluminescence-generating system refers to the components that are necessary and sufficient to generate bioluminescence. These include a  
10 luciferase, luciferin and any necessary co-factors or conditions. Virtually any bioluminescent system known to those of skill in the art will be amenable to use in the apparatus, systems, combinations and methods provided herein. Factors for consideration in selecting a bioluminescent-generating system, include, but are not limited to: the targeting agent used in combination with the  
15 bioluminescence; the medium in which the reaction is run; stability of the components, such as temperature or pH sensitivity; shelf life of the components; sustainability of the light emission, whether constant or intermittent; availability of components; desired light intensity; color of the light; and other such factors.

#### **20 a. General description**

In general, bioluminescence refers to an energy-yielding chemical reaction in which a specific chemical substrate, a luciferin, undergoes oxidation, catalyzed by an enzyme, a luciferase. Bioluminescent reactions are easily maintained, requiring only replenishment of exhausted luciferin or other  
25 substrate or cofactor or other protein, in order to continue or revive the reaction. Bioluminescence generating reactions are well-known to those of skill in this art and any such reaction may be adapted for use in combination with articles of manufacture as described herein.

There are numerous organisms and sources of bioluminescence  
30 generating systems, and some representative genera and species that exhibit bioluminescence are set forth in the following table [reproduced in part from

-46-

Hastings in (1995) *Cell Physiology:Source Book*, N. Sperelakis (ed.), Academic Press, pp 665-681]:

**TABLE 2**  
**Representative luminous organism**

5	Type of Organism	Representative genera
	Bacteria	Photobacterium Vibrio Xenorhabdus
	Mushrooms	Panus, Armillaria Pleurotus
	Dinoflagellates	Gonyaulax Pyrocystis Noctiluca
10	Cnidaria (coelenterates) Jellyfish Hydroid Sea Pansy	Aequorea Obelia Renilla
	Ctenophores	Mnemiopsis Beroe
15	Annelids Earthworms Marine polychaetes Syllid fireworm	Diplocardia Chaetopterus, Phyxotrix Odontosyllis
20	Molluscs Limpet Clam Squid	Latia Pholas Heteroteuthis Heterocarpus
	Crustacea Ostracod	Vargula (Cypridina)
25	Shrimp (euphausids)	Meganyctiphanes Acanthophyra Oplophorus Gnathophausia
30	Decapod Copepods	Sergestes

-47-

	Type of Organism	Representative genera
5	Insects Coleopterids (beetles) Firefly Click beetles Railroad worm Diptera (flies)	Photinus, Photuris Pyrophorus Phengodes, Phrixothrix Arachnocampa
10	Echinoderms Brittle stars Sea cucumbers	Ophiopsila Laetmogone
15	Chordates Tunicates	Pyrosoma
20	Fish Cartilaginous Bony Ponyfish Flashlight fish Angler fish Midshipman Lantern fish Shiny loosejaw Hatchet fish and other fish  Midwater fish	Squalus  Leiognathus Photoblepharon Cryptopsaras Porichthys Benia Aristostomias Agyropelecus Pachystomias Malacosteus  Cyclothone Neoscopelus Tarletonbeania

25 Other bioluminescent organisms contemplated for use herein are *Gonadostomias*, *Gaussia* (copepods), *Watensia*, *Halisturia*, Vampire squid, *Glyphus*, Mycotophids (fish), *Vinciguerria*, *Howella*, *Florenciella*, *Chaudiodus*, *Melanocostus* and Sea Pens.

30 It is understood that a bioluminescence generating system may be isolated from natural sources, such as those in the above Table, or may be produced synthetically. In addition, for uses herein, the components need only be sufficiently pure so that mixture thereof, under appropriate reaction conditions, produces a glow so that cells and tissues can be visualized during a surgical procedure.

-48-

Thus, in some embodiments, a crude extract or merely grinding up the organism may be adequate. Generally, however, substantially pure components are used. Also, components may be synthetic components that are not isolated from natural sources. DNA encoding luciferases is available [see, e.g., SEQ ID Nos. 1-13] and has been modified [see, e.g., SEQ ID Nos. 3 and 10-13] and synthetic and alternative substrates have been devised. The DNA listed herein is only representative of the DNA encoding luciferases that is available.

Any bioluminescence generating system, whether synthetic or isolated from natural sources, such as those set forth in Table 2, elsewhere herein or known to those of skill in the art, is intended for use in the combinations, systems and methods provided herein. Chemiluminescence systems per se, which do not rely on oxygenases [luciferases] are not encompassed herein.

#### (1) Luciferases

The targeted agents herein include luciferases or luciferins. Luciferases refer to any compound that, in the presence of any necessary activators, catalyze the oxidation of a bioluminescence substrate [luciferin] in the presence of molecular oxygen, whether free or bound, from a lower energy state to a higher energy state such that the substrate, upon return to the lower energy state, emits light. For purposes herein, luciferase is broadly used to encompass enzymes that act catalytically to generate light by oxidation of a substrate and also photoproteins, such as aequorin, that act, though not strictly catalytically [since such proteins are exhausted in the reaction], in conjunction with a substrate in the presence of oxygen to generate light. These luciferases, including photoproteins, such as aequorin, are herein also included among the luciferases. These reagents include the naturally-occurring luciferases [including photoproteins], proteins produced by recombinant DNA, and mutated or modified variants thereof that retain the ability to generate light in the presence of an appropriate substrate, co-factors and activators or any other such protein that acts as a catalyst to oxidize a substrate, whereby light is produced.

Generically, the protein that catalyzes or initiates the bioluminescent reaction is referred to as a luciferase, and the oxidizable substrate is referred to as a luciferin. The oxidized reaction product is termed oxyluciferin, and certain

-49-

luciferin precursors are termed etioluciferin. Thus, for purposes herein bioluminescence encompasses light produced by reactions that are catalyzed by [in the case of luciferases that act enzymatically] or initiated by [in the case of the photoproteins, such as aequorin, that are not regenerated in the reaction] a  
5 biological protein or analog, derivative or mutant thereof.

For clarity herein, these catalytic proteins are referred to as luciferases and include enzymes such as the luciferases that catalyze the oxidation of luciferin, emitting light and releasing oxyluciferin. Also included among luciferases are photoproteins, which catalyze the oxidation of luciferin to emit  
10 light but are changed in the reaction and must be reconstituted to be used again. The luciferases may be naturally occurring or may be modified, such as by genetic engineering to improve or alter certain properties. As long as the resulting molecule retains the ability to catalyze the bioluminescent reaction, it is encompassed herein.

15 Any protein that has luciferase activity [a protein that catalyzes oxidation of a substrate in the presence of molecular oxygen to produce light as defined herein] may be used herein. The preferred luciferases are those that are described herein or that have minor sequence variations. Such minor sequence variations include, but are not limited to, minor allelic or species variations and  
20 insertions or deletions of residues, particularly cysteine residues. Suitable conservative substitutions of amino acids are known to those of skill in this art and may be made generally without altering the biological activity of the resulting molecule. Such substitutions are preferably made in accordance with those set forth in TABLE 1 as described above.

25 The luciferases may be obtained commercially, isolated from natural sources, expressed in host cells using DNA encoding the luciferase, or obtained in any manner known to those of skill in the art. For purposes herein, crude extracts obtained by grinding up selected source organisms may suffice. Since large quantities of the luciferase may be desired, isolation of the luciferase from  
30 host cells is preferred. DNA for such purposes is widely available as are modified forms thereof.

-50-

Examples of luciferases include, but are not limited to, those isolated from the ctenophores *Mnemiopsis* (mnemiopsin) and *Beroe ovata* (berovin), those isolated from the coelenterates *Aequorea* (aequorin), *Obelia* (obelin), *Pelagia*, the *Renilla* luciferase, the luciferases isolated from the mollusca *Pholas* (pholasin), the luciferases isolated from fish, such as *Aristostomias*, *Pachystomias* and *Porichthys* and from the ostracods, such as *Cypridina* (also referred to as *Vargula*). Preferred luciferases for use herein are the Aequorin protein, *Renilla* luciferase and *Cypridina* [also called *Vargula*] luciferase [see, e.g., SEQ ID Nos. 1, 2, and 4-13]. Also, preferred are luciferases which react to produce red and/or near infrared light. These include luciferases found in species of *Aristostomias*, such as *A. scintillans*, *Pachystomias*, *Malacosteus*, such as *M. niger*.

## (2) Luciferins

The substrates for the reaction or for inclusion in the conjugates include any molecule(s) with which the luciferase reacts to produce light. Such molecules include the naturally-occurring substrates, modified forms thereof, and synthetic substrates [see, e.g., U.S. Patent Nos. 5,374,534 and 5,098,828]. Exemplary luciferins include those described herein, as well as derivatives thereof, analogs thereof, synthetic substrates, such as dioxetanes [see, e.g., U.S. Patent Nos. 5,004,565 and 5,455,357], and other compounds that are oxidized by a luciferase in a light-producing reaction [see, e.g., U.S. Patent Nos. 5,374,534, 5,098,828 and 4,950,588]. Such substrates also may be identified empirically by selecting compounds that are oxidized in bioluminescent reactions.

## (3) Activators

The bioluminescent generating systems also require additional components discussed herein and known to those of skill in the art. All bioluminescent reactions require molecular oxygen in the form of dissolved or bound oxygen. Thus, molecular oxygen, dissolved in water or in air or bound to a photoprotein, is the activator for bioluminescence reactions. Depending upon the form of the components, other activators include, but are not limited to,



-51-

ATP [for firefly luciferase], flavin reductase [bacterial systems] for regenerating FMNH<sub>2</sub> from FMN, and Ca<sup>2+</sup> or other suitable metal ion [aequorin].

Most of the systems provided herein will generate light when the luciferase and luciferin are mixed and exposed to air or water. The systems that  
5 use photoproteins that have bound oxygen, such as aequorin, however, will require exposure to Ca<sup>2+</sup> [or other suitable metal ion], which can be provided in the form of an aqueous composition of a calcium salt. In these instances, addition of a Ca<sup>2+</sup> [or other suitable metal ion] to a mixture of luciferase [aequorin] and luciferin [such as coelenterazine] will result in generation of light.  
10 The *Renilla* system and other Anthozoa systems also require Ca<sup>2+</sup> [or other suitable metal ion].

If crude preparations are used, such as ground up *Cypridina* [shrimp] or ground fireflies, it may be necessary to add only water. In instances in which fireflies [or a firefly or beetle luciferase] are used the reaction may only require  
15 addition ATP. The precise components will be apparent, in light of the disclosure herein, to those of skill in this art or may be readily determined empirically.

It is also understood that these mixtures will also contain any additional salts or buffers or ions that are necessary for each reaction to proceed. Since  
20 these reactions are well-characterized, those of skill in the art will be able to determine precise proportions and requisite components. Selection of components will depend upon the apparatus, article of manufacture and luciferase. Various embodiments are described and exemplified herein; in view of such description, other embodiments will be apparent.

#### 25 (4) Reactions

In all embodiments, all but one component, either the luciferase or luciferin, of a bioluminescence generating system will be mixed or packaged with or otherwise combined. The remaining component is conjugated to a targeting agent and is intended for administration to an animal.  
30 Prior to a surgical procedure, the conjugate is administered via any suitable route, whereby the targeting agent binds to the targeted tissue by virtue of its specific interaction with a tissue-specific cell surface protein.

-52-

During surgery the tissue is contacted, with the remaining component(s), typically by spraying the area or local injection, and any tissue to which conjugate is bound will glow. The glow should be sufficient to see under dim light or, if necessary, in the dark.

- 5           In general, since the result to be achieved is the production of light visible to the naked eye for qualitative, not quantitative, diagnostic purposes, the precise proportions and amounts of components of the bioluminescence reaction need not be stringently determined or met. They must be sufficient to produce light. Generally, an amount of luciferin and luciferase sufficient to
- 10   generate a visible glow is used; this amount can be readily determined empirically and is dependent upon the selected system and selected application. Where quantitative measurements are required, more precision may be required.

- For purposes herein, such amount is preferably at least the concentrations and proportions used for analytical purposes by those of skill in
- 15   the such arts. Higher concentrations may be used if the glow is not sufficiently bright. Alternatively, a microcarrier coupled to more than one luciferase molecule linked to a targeting agent may be utilized to increase signal output. Also because the conditions in which the reactions are used are not laboratory conditions and the components are subject to storage, higher concentration may
- 20   be used to overcome any loss of activity. Typically, the amounts are 1 mg, preferably 10 mg and more preferably 100 mg, of a luciferase per liter of reaction mixture or 1 mg, preferably 10 mg, more preferably 100 mg. Compositions may contain at least about 0.01 mg/l, and typically 0.1 mg/l, 1 mg/l, 10 mg/l or more of each component on the item. The amount of luciferin
- 25   is also between about 0.01 and 100 mg/l, preferably between 0.1 and 10 mg/l, additional luciferin can be added to many of the reactions to continue the reaction. In embodiments in which the luciferase acts catalytically and does not need to be regenerated, lower amounts of luciferase can be used. In those in which it is changed during the reaction, it also can be replenished; typically
- 30   higher concentrations will be selected. Ranges of concentration per liter [or the amount of coating on substrate the results from contacting with such composition] of each component on the order of 0.1 to 20 mg, preferably 0.1 to

-53-

10 mg, more preferably between about 1 and 10 mg of each component will be sufficient. When preparing coated substrates, as described herein, greater amounts of coating compositions containing higher concentrations of the luciferase or luciferin may be used.

- 5           Thus, for example, in presence of calcium, 5 mg of luciferin, such as coelenterazine, in one liter of water will glow brightly for at least about 10 to 20 minutes, depending on the temperature of the water, when about 10 mgs of luciferase, such as aequorin photoprotein luciferase or luciferase from *Renilla*, is added thereto. Increasing the concentration of luciferase, for example, to 100  
10 mg/l, provides a particularly brilliant display of light.

- It is understood, that concentrations and amounts to be used depend upon the selected bioluminescence generating system but these may be readily determined empirically. Proportions, particularly those used when commencing an empirical determination, are generally those used for analytical purposes, and  
15 amounts or concentrations are at least those used for analytical purposes, but the amounts can be increased, particularly if a sustained and brighter glow is desired.

## 2.     The *Renilla* system

- Renilla*, also known as soft coral sea pansies, are members of the class  
20 of coelenterates Anthozoa, which includes other bioluminescent genera, such as *Cavarnularia*, *Ptilosarcus*, *Stylatula*, *Acanthoptilum*, and *Parazoanthus*. Bioluminescent members of the Anthozoa genera contain luciferases and luciferins that are similar in structure [see, e.g., Cormier et al. (1973) J. Cell. Physiol. 81:291-298; see, also Ward et al. (1975) Proc. Natl. Acad. Sci. U.S.A.  
25 72:2530-2534]. The luciferases and luciferins from each of these anthozoans crossreact with one another and produce a characteristic blue luminescence.

- Renilla* luciferase and the other coelenterate and ctenophore luciferases, such as the aequorin photoprotein, use imidazopyrazine substrates, particularly the substrates generically called coelenterazine [see, formulae (I) and (II) of  
30 Section B.1.b, above]. Other genera that have luciferases that use a coelenterazine include: squid, such as *Chroteuthis*, *Eucleoteuthis*, *Onychoteuthis*, *Watasenia*, cuttlefish, *Sepiolina*; shrimp, such as *Oplophorus*,

-54-

*Acanthophyra*, *Sergestes*, and *Gnathophausia*; deep-sea fish, such as *Argyrolepecus*, *Yarella*, *Diaphus*, *Gonadostomias* and *Neoscopelus*.

*Renilla* luciferase does not, however, have bound oxygen, and thus requires dissolved oxygen in order to produce light in the presence of a suitable luciferin substrate. Since *Renilla* luciferase acts as a true enzyme [i.e., it does not have to be reconstituted for further use] the resulting luminescence can be long-lasting in the presence of saturating levels of luciferin. Also, *Renilla* luciferase is relatively stable to heat.

*Renilla* luciferases, DNA encoding *Renilla reniformis* luciferase, and use of the *Renilla reniformis* DNA to produce recombinant luciferase, as well as DNA encoding luciferase from other coelenterates, are well known and available [see, e.g., SEQ ID No. 1, U.S. Patent Nos. 5,418,155 and 5,292,658; see, also, Prasher et al. (1985) Biochem. Biophys. Res. Commun. 126:1259-1268; Cormier (1981) "Renilla and Aequorea bioluminescence" in Bioluminescence and Chemiluminescence, pp. 225-233; Charbonneau et al. (1979) J. Biol. Chem. 254:769-780; Ward et al. (1979) J. Biol. Chem. 254:781-788; Lorenz et al. (1981) Proc. Natl. Acad. Sci. U.S.A. 88: 4438-4442; Hori et al. (1977) Proc. Natl. Acad. Sci. U.S.A. 74:4285-4287; Hori et al. (1975) Biochemistry 14:2371-2376; Hori et al. (1977) Proc. Natl. Acad. Sci. U.S.A. 74:4285-4287; Inouye et al. (1975) Jap. Soc. Chem. Lett. 141-144; and Matthews et al. (1979) Biochemistry 16:85-91]. The DNA encoding *Renilla reniformis* luciferase and host cells containing such DNA provide a convenient means for producing large quantities of *Renilla reniformis* enzyme, such as in those known to those of skill in the art [see, e.g., U.S. Patent Nos. 5,418,155 and 5,292,658, which describe recombinant production of *Renilla reniformis* luciferase].

When used herein, the *Renilla* luciferase can be packaged in lyophilized form, encapsulated in a vehicle, either by itself or in combination with the luciferin substrate. Prior to use the mixture is contacted with an aqueous composition, preferably a phosphate buffered saline pH 7-8; dissolved O<sub>2</sub> will activate the reaction. Final concentrations of luciferase in the glowing mixture will be on the order of 0.01 to 1 mg/l or more. Concentrations of luciferin will

-55-

be at least about  $10^{-8}$  M, but 1 to 100 or more orders of magnitude higher to produce a long lasting bioluminescence.

In certain embodiments herein, about 1 to 10 mg, or preferably 2-5 mg, more preferably about 3 mg of coelenterazine will be used with about 100 mg of *Renilla* luciferase. The precise amounts, of course can be determined empirically, and, also will depend to some extent on the ultimate concentration and application. In particular, about addition of about 0.25 ml of a crude extract from the bacteria that express *Renilla* to 100 ml of a suitable assay buffer and about 0.005  $\mu$ g was sufficient to produce a visible and lasting glow [see, U.S. Patent Nos. 5,418,155 and 5,292,658, which describe recombinant production of *Renilla reniformis* luciferase].

Lyophilized mixtures, and compositions containing the *Renilla* luciferase are also provided. The luciferase or mixtures of the luciferase and luciferin may also be encapsulated into a suitable delivery vehicle, such as a liposome, glass particle, capillary tube, drug delivery vehicle, gelatin, time release coating or other such vehicle. The luciferase may also be linked to a substrate, such as biocompatible materials.

#### b. Ctenophore systems

Ctenophores, such as *Mnemiopsis* (mnemiopsin) and *Beroe ovata* (berovin), and coelenterates, such as *Aequorea* (aequorin), *Obelia* (obelin) and *Pelagia*, produce bioluminescent light using similar chemistries [see, e.g., Stephenson et al. (1981) *Biochimica et Biophysica Acta* 678:65-75; Hart et al. (1979) *Biochemistry* 18:2204-2210; International PCT Application No. WO 94/18342, which is based on U.S. application Serial No. 08/017,116, U.S. Patent No. 5,486,455 and other references and patents cited herein]. The *Aequorin* and *Renilla* systems are representative and are described in detail herein as exemplary and as among the presently preferred systems. The *Aequorin* and *Renilla* systems can use the same luciferin and produce light using the same chemistry, but each luciferase is different. The *Aequorin* luciferase aequorin, as well as, for example, the luciferases mnemiopsin and berovin, is a photoprotein that includes bound oxygen and bound luciferin, requires  $\text{Ca}^{2+}$  [or other suitable metal ion] to trigger the reaction, and must be regenerated for

-56-

repeated use; whereas, the *Renilla* luciferase acts as a true enzyme because it is unchanged during the reaction and it requires dissolved molecular oxygen.

(1) The aequorin system

The aequorin system is well known [see, e.g., Tsuji et al. (1986)

- 5 "Site-specific mutagenesis of the calcium-binding photoprotein aequorin," Proc. Natl. Acad. Sci. USA 83:8107-8111; Prasher et al. (1985) "Cloning and Expression of the cDNA Coding for Aequorin, a Bioluminescent Calcium-Binding Protein," Biochemical and Biophysical Research Communications 126:1259-1268; Prasher et al. (1986) Methods in Enzymology 133:288-297;
- 10 Prasher, et al. (1987) "Sequence Comparisons of cDNAs Encoding for Aequorin Isotypes," Biochemistry 26:1326-1332; Charbonneau et al. (1985) "Amino Acid Sequence of the Calcium-Dependent Photoprotein Aequorin," Biochemistry 24:6762-6771; Shimomura et al. (1981) "Resistivity to denaturation of the apoprotein of aequorin and reconstitution of the luminescent
- 15 photoprotein from the partially denatured apoprotein," Biochem. J. 199:825-828; Inouye et al. (1989) J. Biochem. 105:473-477; Inouye et al. (1986) "Expression of Apoequorin Complementary DNA in *Escherichia coli*," Biochemistry 25:8425-8429; Inouye et al. (1985) "Cloning and sequence analysis of cDNA for the luminescent protein aequorin," Proc. Natl. Acad. Sci.
- 20 USA 82:3154-3158; Prendergast, et al. (1978) "Chemical and Physical Properties of Aequorin and the Green Fluorescent Protein Isolated from *Aequorea forskalea*" J. Am. Chem. Soc. 100:3448-3453; European Patent Application 0 540 064 A1; European Patent Application 0 226 979 A2, European Patent Application 0 245 093 A1 and European Patent Application 0
- 25 245 093 B1; U.S. Patent No. 5,093,240; U.S. Patent No. 5,360,728; U.S. Patent No. 5,139,937; U.S. Patent No. 5,422,266; U.S. Patent No. 5,023,181; U.S. Patent No. 5,162,227; and SEQ ID Nos. 5-13, which set forth DNA encoding the apoprotein; and a form, described in U.S. Patent No. 5,162,227, European Patent Application 0 540 064 A1 and Sealite Sciences Technical
- 30 Report No. 3 (1994), is commercially available from Sealite, Sciences, Bogart, GA as AQUALITE®].

-57-

This system is among the preferred systems for use herein. As will be evident, since the aequorin photoprotein includes noncovalently bound luciferin and molecular oxygen, it is suitable for storage in this form as a lyophilized powder or encapsulated into a selected delivery vehicle. The system can be  
5 encapsulated into pellets, such as liposomes or other delivery vehicles. When used, the vehicles are contacted with a composition, even tap water, that contains  $\text{Ca}^{2+}$  [or other suitable metal ion], to produce a mixture that glows.

(a) Aequorin and related photoproteins

The photoprotein, aequorin, isolated from the jellyfish, *Aequorea*, emits  
10 light upon the addition of  $\text{Ca}^{2+}$  [or other suitable metal ion]. The aequorin photoprotein, which includes bound luciferin and bound oxygen that is released by  $\text{Ca}^{2+}$ , does not require dissolved oxygen. Luminescence is triggered by calcium, which releases oxygen and the luciferin substrate producing apoaequorin.

15 The bioluminescence photoprotein aequorin is isolated from a number of species of the jellyfish *Aequorea*. It is a 22 kilodalton [kD] molecular weight peptide complex [see, e.g., Shimomura *et al.* (1962) *J. Cellular and Comp. Physiol.* 59:233-238; Shimomura *et al.* (1969) *Biochemistry* 8:3991-3997; Kohama *et al.* (1971) *Biochemistry* 10:4149-4152; and Shimomura *et al.* (1972)  
20 *Biochemistry* 11:1602-1608]. The native protein contains oxygen and a heterocyclic compound coelenterazine, a luciferin, [see, below] noncovalently bound thereto. The protein contains three calcium binding sites. Upon addition of trace amounts  $\text{Ca}^{2+}$  [or other suitable metal ion, such as strontium] to the photoprotein, it undergoes a conformational change that catalyzes the oxidation  
25 of the bound coelenterazine using the protein-bound oxygen. Energy from this oxidation is released as a flash of blue light, centered at 469 nm. Concentrations of calcium ions as low as  $10^{-6}$  M are sufficient to trigger the oxidation reaction.

Naturally-occurring apoaequorin is not a single compound but rather is a  
30 mixture of microheterogeneous molecular species. *Aequoria* jellyfish extracts contain as many as twelve distinct variants of the protein [see, e.g., Prasher *et al.* (1987) *Biochemistry* 26:1326-1332; Blinks *et al.* (1975) *Fed. Proc.* 34:474].

-58-

DNA encoding numerous forms has been isolated [see, e.g., SEQ ID Nos. 5-9 and 13].

The photoprotein can be reconstituted [see, e.g., U.S. Patent No. 5,023,181] by combining the apoprotein, such as a protein recombinantly  
5 produced in E. coli, with a coelenterazine, such as a synthetic coelenterazine, in the presence of oxygen and a reducing agent [see, e.g., Shimomura et al. (1975) Nature 256:236-238; Shimomura et al. (1981) Biochemistry J. 199:825-828], such as 2-mercaptoethanol, and also EDTA or EGTA [concentrations  
10 between about 5 to about 100 mM or higher for applications herein] tie up any  $\text{Ca}^{2+}$  to prevent triggering the oxidation reaction until desired. DNA encoding a modified form of the apoprotein that does not require 2-mercaptoethanol for reconstitution is also available [see, e.g., U.S. Patent No. U.S. Patent No. 5,093,240]. The reconstituted photoprotein is also commercially available [sold,  
15 e.g., under the trademark AQUALITE<sup>®</sup>, which is described in U.S. Patent No. 5,162,227].

The light reaction is triggered by adding  $\text{Ca}^{2+}$  at a concentration sufficient to overcome the effects of the chelator and achieve the  $10^{-6}$  M concentration. Because such low concentrations of  $\text{Ca}^{2+}$  can trigger the reaction, for use in the methods herein, higher concentrations of chelator may  
20 be included in the compositions of photoprotein. Accordingly, higher concentrations of added  $\text{Ca}^{2+}$  in the form of a calcium salt will be required. Precise amounts may be empirically determined. For use herein, it may be sufficient to merely add water to the photoprotein, which is provided in the form of a concentrated composition or in lyophilized or powdered form. Thus,  
25 for purposes herein, addition of small quantities of  $\text{Ca}^{2+}$ , such as those present in phosphate buffered saline (PBS) or other suitable buffers or the moisture on the tissue to which the compositions are contacted, should trigger the bioluminescence reaction.

Numerous isoforms of the aequorin apoprotein been identified isolated.  
30 DNA encoding these proteins has been cloned, and the proteins and modified forms thereof have been produced using suitable host cells [see, e.g., U.S. Patent Nos. 5,162,227, 5,360,728, 5,093,240; see, also, Prasher et al. (1985)



-59-

Biophys. Biochem. Res. Commun. 126:1259-1268; Inouye et al. (1986) Biochemistry 25: 8425-8429]. U.S. Patent No. 5,093,240; U.S. Patent No. 5,360,728; U.S. Patent No. 5,139,937; U.S. Patent No. 5,288,623; U.S. Patent No. 5,422,266, U.S. Patent No. 5,162,227 and SEQ ID Nos. 5-13, which set forth DNA encoding the apoprotein; and a form is commercially available from Sealite, Sciences, Bogart, GA as AQUALITE®]. DNA encoding apoaequorin or variants thereof is useful for recombinant production of high quantities of the apoprotein. The photoprotein is reconstituted upon addition of the luciferin, coelenterazine, preferably a sulfated derivative thereof, or an analog thereof, and molecular oxygen [see, e.g., U.S. Patent No. 5,023,181]. The apoprotein and other constituents of the photoprotein and bioluminescence generating reaction can be mixed under appropriate conditions to regenerate the photoprotein and concomitantly have the photoprotein produce light. Reconstitution requires the presence of a reducing agent, such as mercaptoethanol, except for modified forms, discussed below, that are designed so that a reducing agent is not required [see, e.g., U.S. Patent No. 5,093,240].

For use herein, it is preferred aequorin is produced using DNA, such as that set forth in SEQ ID Nos. 5-13 and known to those of skill in the art or modified forms thereof. The DNA encoding aequorin is expressed in a host cell, such as E. coli, isolated and reconstituted to produce the photoprotein [see, e.g., U.S. Patent Nos. 5,418,155, 5,292,658, 5,360,728, 5,422,266, 5,162,227].

Of interest herein, are forms of the apoprotein that have been modified so that the bioluminescent activity is greater than unmodified apoaequorin [see, e.g., U.S. Patent No. 5,360,728, SEQ ID Nos. 10-12]. Modified forms that exhibit greater bioluminescent activity than unmodified apoaequorin include proteins including sequences set forth in SEQ ID Nos. 10-12, in which aspartate 124 is changed to serine, glutamate 135 is changed to serine, and glycine 129 is changed to alanine, respectively. Other modified forms with increased bioluminescence are also available.

For use in certain embodiments herein, the apoprotein and other components of the aequorin bioluminescence generating system are packaged

-60-

or provided as a mixture, which, when desired is subjected to conditions under which the photoprotein reconstitutes from the apoprotein, luciferin and oxygen [see, e.g., U.S. Patent No. 5,023,181; and U.S. Patent No. 5,093,240].

Particularly preferred are forms of the apoprotein that do not require a reducing agent, such as 2-mercaptoethanol, for reconstitution. These forms, described, for example in U.S. Patent No. 5,093,240 [see, also Tsuji et al. (1986) Proc. Natl. Acad. Sci. U.S.A. 83:8107-8111], are modified by replacement of one or more, preferably all three cysteine residues with, for example serine.

Replacement may be effected by modification of the DNA encoding the aequorin apoprotein, such as that set forth in SEQ ID No. 5, and replacing the cysteine codons with serine.

The photoproteins and luciferases from related species, such as *Obelia* are also contemplated for use herein. DNA encoding the  $\text{Ca}^{2+}$ -activated photoprotein obelin from the hydroid polyp *Obelia longissima* is known and available [see, e.g., Illarionov et al. (1995) Gene 153:273-274; and Bondar et al. (1995) Biochim. Biophys. Acta 1231:29-32]. This photoprotein can also be activated by  $\text{Mn}^{2+}$  [see, e.g., Vysotski et al. (1995) Arch. Bioch. Biophys. 316:92-93, Vysotski et al. (1993) J. Biolumin. Chemilumin. 8:301-305].

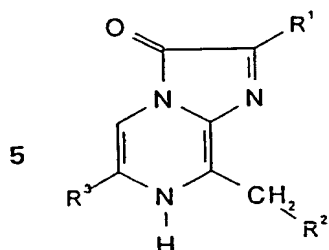
In general for use herein, the components of the bioluminescence are packaged or provided so that there is insufficient metal ions to trigger the reaction. When used, the trace amounts of triggering metal ion, particularly  $\text{Ca}^{2+}$  is contacted with the other components. For a more sustained glow, aequorin can be continuously reconstituted or can be added or can be provided in high excess.

#### 25 (b) Luciferin

The aequorin luciferin is coelenterazine and analogs therein, which include molecules including the structure [formula (I)]:

30

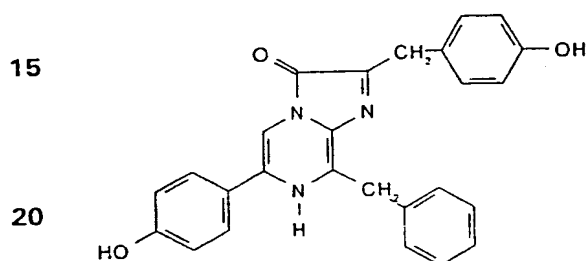
-61-



in which R<sub>1</sub> is CH<sub>2</sub>C<sub>6</sub>H<sub>5</sub> or CH<sub>3</sub>; R<sub>2</sub> is C<sub>6</sub>H<sub>5</sub>, and R<sub>3</sub> is *p*-C<sub>6</sub>H<sub>4</sub>OH or CH<sub>3</sub> or other such analogs that have activity. Preferred coelenterazine has the structure in which R<sub>1</sub> is *p*-CH<sub>2</sub>C<sub>6</sub>H<sub>4</sub>OH, R<sub>2</sub> is C<sub>6</sub>H<sub>5</sub>, and R<sub>3</sub> is *p*-C<sub>6</sub>H<sub>4</sub>OH, which can be prepared by known methods [see, e.g., Inouye *et al.* (1975) *Jap. Chem. Soc.,*

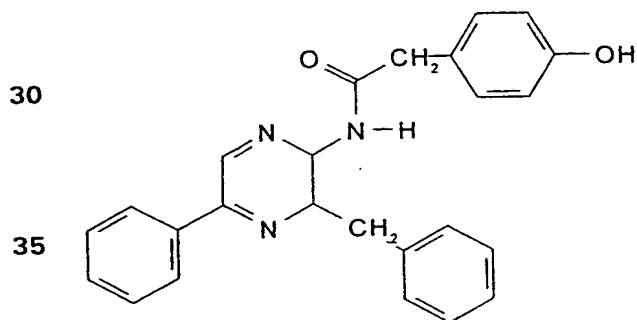
10 *Chemistry Ltrts.* pp 141-144; and Hart *et al.* (1979) *Biochemistry* 18:2204-2210]. Among the preferred analogs, are those that are modified, whereby the spectral frequency of the resulting light is shifted to another frequency.

The preferred coelenterazine has the structure (formula (II)):



and sulfated derivatives thereof.

25 Another coelenterazine has formula (V):



40

[see, Hart *et al.* (1979)

*Biochemistry* 18:2204-2210]. Using this derivative in the presence of

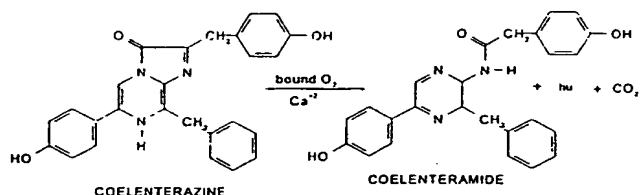
-62-

luciferase all of the light is in the ultraviolet with a peak at 390 nm. Upon addition of GFP, all light emitted is now in the visible range with a peak at 509 nm accompanied by an about 200-fold increase in the amount of light emitted. Viewed with a cut-off filter of 470 nm, in the light yield in the absence of GFP would be about zero, and would be detectable in the presence of GFP. This provides the basis for an immunoassay described in the EXAMPLES.

The reaction of coelenterazine when bound to the aequorin photoprotein with bound oxygen and in the presence of  $\text{Ca}^{2+}$  can be represented as follows:

10

15



20

The photoprotein aequorin [which contains apoaequorin bound to a coelenterate luciferin molecule] and *Renilla* luciferase, discussed below, can use the same coelenterate luciferin. The aequorin photoprotein catalyses the oxidation of coelenterate luciferin [coelenterazine] to oxyluciferin [coelenteramide] with the concomitant production of blue light [ $\lambda_{\text{max}} = 469 \text{ nm}$ ].

30

Importantly, the sulfate derivative of the coelenterate luciferin [lauryl-luciferin] is particularly stable in water, and thus may be used in a coelenterate-like bioluminescent system. In this system, adenosine diphosphate (ADP) and a sulpha-kinase are used to convert the coelenterazine to the sulphated form. Sulfatase is then used to reconvert the lauryl-luciferin to the native

35

coelenterazine. Thus, the more stable lauryl-luciferin is used in the item to be illuminated and the luciferase combined with the sulfatase are added to the luciferin mixture when illumination is desired.

-63-

Thus, the bioluminescent system of *Aequorea* is particularly suitable for use in the methods herein. The particular amounts and the manner in which the components are provided depends upon the type of neoplasia or specialty tissue to be visualized. This system can be provided in lyophilized form, that will glow upon addition of  $\text{Ca}^{2+}$ . It can be encapsulated, linked to microcarriers, such as microbeads, or in as a compositions, such as a solution or suspension, preferably in the presence of sufficient chelating agent to prevent triggering the reaction. The concentration of the aequorin photoprotein will vary and can be determined empirically. Typically concentrations of at least 0.1 mg/l, more preferably at least 1 mg/l and higher, will be selected. In certain embodiments, 1-10 mg luciferin/100 mg of luciferase will be used in selected volumes and at the desired concentrations will be used.

c. Crustacean, particularly *Cypridina* systems

The ostracods, such as *Vargula serrata*, *hilgendorfii* and *noctiluca* are small marine crustaceans, sometimes called sea fireflies. These sea fireflies are found in the waters off the coast of Japan and emit light by squirting luciferin and luciferase into the water, where the reaction, which produces a bright blue luminous cloud, occurs. The reaction involves only luciferin, luciferase and molecular oxygen, and, thus, is very suitable for application herein.

The systems, such as the *Vargula* bioluminescent systems, are particularly preferred herein because the components are stable at room temperature if dried and powdered and will continue to react even if contaminated. Further, the bioluminescent reaction requires only the luciferin/luciferase components in concentrations as low as 1:40 parts per billion to 1:100 parts per billion, water and molecular oxygen to proceed. An exhausted system can renewed by addition of luciferin.

(1) *Vargula* luciferase

The *Vargula* luciferase is water soluble and is among those preferred for use in the methods herein. *Vargula* luciferase is a 555-amino acid polypeptide that has been produced by isolation from *Vargula* and also using recombinant technology by expressing the DNA in suitable bacterial and mammalian host cells [see, e.g., Thompson *et al.* (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86:6567-

-64-

6571; Inouye et al. (1992) Proc. Natl. Acad. Sci. U.S.A. 89:9584-9587;  
Johnson et al. (1978) Methods in Enzymology LVI:331-349; Tsuji et al. (1978)  
Methods Enzymol. 57:364-72; Tsuji (1974) Biochemistry 13:5204-5209;  
Japanese Patent Application No. JP 3-30678 Osaka; and European Patent  
5 Application No. EP 0 387 355 A1].

(a) Purification from *Cypridina*

Methods for purification of *Vargula* [*Cypridina*] luciferase are well known.  
For example, crude extracts containing the active can be  
readily prepared by grinding up or crushing the *Vargula* shrimp. In other  
10 embodiments, a preparation of *Cypridina hilgendorfi* luciferase can be prepared  
by immersing stored frozen *C. hilgendorfi* in distilled water containing, 0.5-5.0  
M salt, preferably 0.5-2.0 M sodium or potassium chloride, ammonium sulfate,  
at 0-30° C, preferably 0-10° C, for 1-48 hr, preferably 10-24 hr, for extraction  
followed by hydrophobic chromatography and then ion exchange or affinity  
15 chromatography [TORAY IND INC, Japanese patent application JP 4258288,  
published September 14, 1993; see, also, Tsuji et al. (1978) Methods Enzymol.  
57:364-72 for other methods].

(b) Preparation by Recombinant Methods

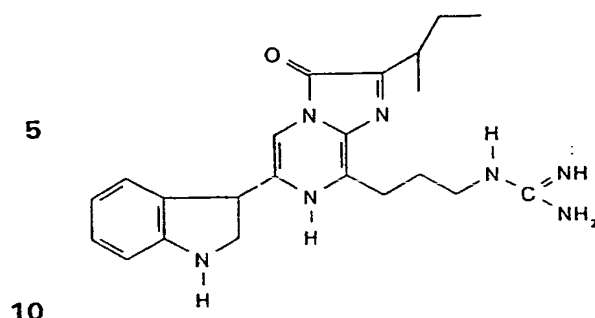
The luciferase is preferably produced by expression of cloned DNA  
20 encoding the luciferase [European Patent Application No. 0 387 355 A1;  
International PCT Application No. WO 95/001542; see, also SEQ ID No. 5,  
which sets forth the sequence from Japanese Patent Application No. JP 3-  
30678 and Thompson et al. (1989) Proc. Natl. Acad. Sci. U.S.A. 86:6567-  
6571] DNA encoding the luciferase or variants thereof is introduced into *E. coli*  
25 using appropriate vectors and isolated using standard methods.

(2) *Vargula* luciferin

The natural luciferin is a substituted imidazopyrazine nucleus, such a  
compound of formula (III):

30

-65-



The luciferin can be isolated from ground dried *Vargula* by heating the extract, which destroys the luciferase but leaves the

luciferin intact [see, e.g., U.S. Patent No. 4,853,327].

Analogous thereof and other compounds that react with the luciferase in a light producing reaction also may be used.

15 Other bioluminescent organisms that have luciferases that can react with the *Vargula* luciferin include, the genera *Apogon*, *Parapriacanthus* and *Porichthys*.

### (3) Reaction

20 The luciferin upon reaction with oxygen forms a dioxetanone intermediate [which includes a cyclic peroxide similar to the firefly cyclic peroxide molecule intermediate]. In the final step of the bioluminescent reaction, the peroxide breaks down to form CO<sub>2</sub> and an excited carbonyl. The excited molecule then emits a blue to blue-green light.

25 The optimum pH for the reaction is about 7. For purposes herein, any pH at which the reaction occurs may be used. The concentrations of reagents are those normally used for analytical reactions or higher [see, e.g., Thompson et al. (1990) Gene 96:257-262]. Typically concentrations of the luciferase between 0.1 and 10 mg/l, preferably 0.5 to 2.5 mg/l will be used. Similar concentrations or higher concentrations of the luciferin may be used.

30 d. Insect bioluminescent systems including fireflies, click beetles, and other insect system

The biochemistry of firefly bioluminescence was the first bioluminescent system to be characterized [see, e.g.,

-66-

- Wienhausen et al. (1985) Photochemistry and Photobiology 42:609-611;  
 McElroy et al. (1966) in Molecular Architecture in cell Physiology, Hayashi et al., eds. Prentice Hall, Inc., Englewood Cliffs, NJ, pp. 63-80] and it is commercially available [e.g., from Promega Corporation, Madison, WI, see, e.g.,  
 5 Leach et al. (1986) Methods in Enzymology 133:51-70, esp. Table 1].  
 Luciferases from different species of fireflies are antigenically similar. These species include members of the genera *Photinus*, *Photurins* and *Luciola*. Further, the bioluminescent reaction produces more light at 30°C than at 20°C, the luciferase is stabilized by small quantities of bovine albumin serum, and the  
 10 reaction can be buffered by tricine.

## (1) Luciferase

- DNA clones encoding luciferases from various insects and the use to produce the encoded luciferase is well known. For example, DNA clones that encode luciferase from *Photinus pyralis*, *Luciola cruciata* [see, e.g., de Wet et al.  
 15 (1985) Proc. Natl. Acad. Sci. U.S.A. 82:7870-7873; de We et al. (1986) Methods in Enzymology 133:3; U.S. Patent No. 4,968,613, see, also SEQ ID No. 3] are available. The DNA has also been expressed in *Saccharomyces* [see, e.g., Japanese Application No. JP 63317079, published December 26, 1988, KIKKOMAN CORP] and in tobacco.

- 20 In addition to the wild-type luciferase modified insect luciferases have been prepared. For example, heat stable luciferase mutants, DNA-encoding the mutants, vectors and transformed cells for producing the luciferases are available. A protein with 60% amino acid sequence homology with luciferases from *Photinus pyralis*, *Luciola mingrelica*, *L. cruciata* or *L. lateralis* and having  
 25 luciferase activity is available [see, e.g., International PCT Application No. WO 95/25798]. It is more stable above 30° C than naturally-occurring insect luciferases and may also be produced at 37° C or above, with higher yield.

- Modified luciferases that generate light at different wavelengths [compared with native luciferase], and thus, may be selected for their color-  
 30 producing characteristics. For example, synthetic mutant beetle luciferase(s) and DNA encoding such luciferases that produce bioluminescence at a wavelength different from wild-type luciferase are known [Promega Corp,



International PCT Application No. WO 95/18853, which is based on U.S. application Serial No. 08/177,081]. The mutant beetle luciferase has an amino acid sequence differing from that of the corresponding wild-type *Luciola cruciata* [see, e.g., U.S. Patent Nos. 5,182,202, 5,219,737, 5,352,598, see, also SEQ ID No.3] by a substitution(s) at one or two positions. The mutant luciferase produces a bioluminescence with a wavelength of peak intensity that differs by at least 1 nm from that produced by wild-type luciferases.

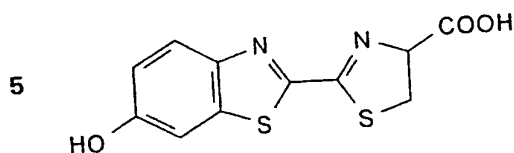
Other mutant luciferases can be produced. Mutant luciferases with the amino acid sequence of wild-type luciferase, but with at least one mutation in which valine is replaced by isoleucine at the amino acid number 233, valine by isoleucine at 239, serine by asparagine at 286, glycine by serine at 326, histidine by tyrosine at 433 or proline by serine at 452 are known [see, e.g., U.S. Patent Nos. 5,219,737, and 5,330,906]. The luciferases are produced by expressing DNA-encoding each mutant luciferase in *E. coli* and isolating the protein. These luciferases produce light with colors that differ from wild-type. The mutant luciferases catalyze luciferin to produce red ( $\lambda$  609 nm and 612 nm), orange ( $\lambda$  595 and 607 nm) or green ( $\lambda$  558 nm) light. The other physical and chemical properties of mutant luciferase are substantially identical to native wild type-luciferase. The mutant luciferase has the amino acid sequence of *Luciola cruciata* luciferase with an alteration selected from Ser 286 replaced by Asn, Gly 326 replaced by Ser, His 433 replaced by Tyr or Pro 452 replaced by Ser. Thermostable luciferases are also available [see, e.g., U.S. Patent No. 5,229,285; see, also International PCT Application No. WO 95/25798, which provides *Photinus* luciferase in which the glutamate at position 354 is replaced with lysine and *Luciola* luciferase in which the glutamate at 356 is replaced with lysine].

These mutant luciferases as well as the wild type luciferases can be used in combination with the GFPs provided herein particularly in instances when a variety of colors are desired or when stability at higher temperatures is desired.

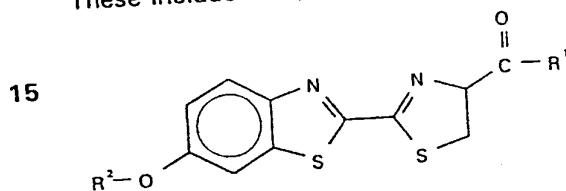
-68-

## (2) Luciferin

The firefly luciferin is a benzothiazole:



10      Analogs of this luciferin and synthetic firefly luciferins are also known to those of skill in art [see, *e.g.*, U.S. Patent No. 5,374,534 and 5,098,828]. These include compounds of formula (IV) [see, U.S. Patent No. 5,098,828]:



in which:

- 20      R<sup>1</sup> is hydroxy, amino, linear or branched C<sub>1</sub>-C<sub>20</sub> alkoxy, C<sub>2</sub>-C<sub>20</sub> alkenyloxy, an L-amino acid radical bond via the α-amino group, an oligopeptide radical with up to ten L-amino acid units linked via the α-amino group of the terminal unit;
- 25      R<sup>2</sup> is hydrogen, H<sub>2</sub>PO<sub>3</sub>, HSO<sub>3</sub>, unsubstituted or phenyl substituted linear or branched C<sub>1</sub>-C<sub>20</sub> alkyl or C<sub>2</sub>-C<sub>20</sub> alkenyl, aryl containing 6 to 18 carbon atoms, or R<sup>3</sup>-C(O)-; and
- 30      R<sup>3</sup> is an unsubstituted or phenyl substituted linear or branched C<sub>1</sub>-C<sub>20</sub> alkyl or C<sub>2</sub>-C<sub>20</sub> alkenyl, aryl containing 6 to 18 carbon atoms, a nucleotide radical with 1 to 3 phosphate groups, or a glycosidically attached mono- or disaccharide, except when formula (IV) is a D-luciferin or D-luciferin methyl ester.

Modified luciferins that have been modified to produce light of shifted frequencies are known to those of skill in the art.

## (3) Reaction

35      The reaction catalyzed by firefly luciferases and related insect luciferases requires ATP, Mg<sup>2+</sup> as well as molecular oxygen. Luciferin must be added

-69-

exogenously. Firefly luciferase catalyzes the firefly luciferin activation and the subsequent steps leading to the excited product. The luciferin reacts with ATP to form a luciferyl adenylate intermediate. This intermediate then reacts with oxygen to form a cyclic luciferyl peroxy species, similar to that of the  
5 coelenterate intermediate cyclic peroxide, which breaks down to yield CO<sub>2</sub> and an excited state of the carbonyl product. The excited molecule then emits a yellow light; the color, however, is a function of pH. As the pH is lowered the color of the bioluminescence changes from yellow-green to red.

Different species of fireflies emit different colors of bioluminescence so  
10 that the color of the reaction will be dependent upon the species from which the luciferase is obtained. Additionally, the reaction is optimized at pH 7.8.

Addition of ATP and luciferin to a reaction that is exhausted produces additional light emission. Thus, the system, once established, is relatively easily maintained. Therefore, it is highly suitable for use herein in embodiments in  
15 which a sustained glow is desired.

e. Bacterial systems

Luminous bacteria typically emit a continuous light, usually blue-green. When strongly expressed, a single bacterium may emit 10<sup>4</sup> to 10<sup>5</sup> photons per second. Bacterial bioluminescence systems include, among others, those  
20 systems found in the bioluminescent species of the genera *Photobacterium*, *Vibrio* and *Xenorhabdus*. These systems are well known and well characterized [see, e.g., Baldwin *et al.* (1984) *Biochemistry* 23:3663-3667; Nicoli *et al.* (1974) *J. Biol. Chem.* 249:2393-2396; Welches *et al.* (1981) *Biochemistry* 20:512-517; Engebrecht *et al.* (1986) *Methods in Enzymology* 133:83-99;  
25 Frackman *et al.* (1990) *J. of Bacteriology* 172:5767-5773; Miyamoto *et al.* (1986) *Methods in Enzymology* 133:70; U.S. Patent No. 4,581,335].

(1) Luciferases

Bacterial luciferase, as exemplified by luciferase derived from *Vibrio harveyi* [EC 1.14.14.3, alkanol reduced-FMN-oxygen oxidoreductase 1-hydroxylating,  
30 luminescing], is a mixed function oxidase, formed by the association of two different protein subunits  $\alpha$  and  $\beta$ . The  $\alpha$ -subunit has an apparent molecular weight of approximately 42,000 kD and the  $\beta$ -subunit has an apparent

-70-

molecular weight of approximately 37,000 kD [see, e.g., Cohn et al. (1989) Proc. Natl. Acad. Sci. U.S.A. 90:102-123]. These subunits associate to form a 2-chain complex luciferase enzyme, which catalyzes the light emitting reaction of bioluminescent bacteria, such as *Vibrio harveyi* [U.S. Patent No. 4,581,335; 5 Belas et al. (1982) Science 218:791-793], *Vibrio fischeri* [Engebrecht et al. (1983) Cell 32:773-781; Engebrecht et al. (1984) Proc. Natl. Acad. Sci. U.S.A. 81:4154-4158] and other marine bacteria.

Bacterial luciferase genes have been cloned [see, e.g., U.S. Patent No. 5,221,623; U.S. Patent No. 4,581,335; European Patent Application No. EP 10 386 691 A]. Plasmids for expression of bacterial luciferase, such as *Vibrio harveyi*, include pFIT001 (NRRL B-18080), pPALE001 (NRRL B-18082) and pMR19 (NRRL B-18081)] are known. For example the sequence of the entire *lux* regulon from *Vibrio fischeri* has been determined [Baldwin et al. (1984), Biochemistry 23:3663-3667; Baldwin et al. (1981) Biochem. 20: 512-517; 15 Baldwin et al. (1984) Biochem. 23:3663-3667; see, also, e.g., U.S. Patent Nos. 5,196,318, 5,221,623, and 4,581,335]. This regulon includes *luxI* gene, which encodes a protein required for autoinducer synthesis [see, e.g., Engebrecht et al. (1984) Proc. Natl. Acad. Sci. U.S.A. 81:4154-4158], the *luxC*, *luxD*, and *luxE* genes, which encode enzymes that provide the luciferase 20 with an aldehyde substrate, and the *luxA* and *luxB* genes, which encode the alpha and beta subunits of the luciferase.

Lux genes from other bacteria have also been cloned and are available [see, e.g., Cohn et al. (1985) J. Biol. Chem. 260:6139-6146; U.S. Patent No. 5,196,524, which provides a fusion of the *luxA* and *luxB* genes from *Vibrio* 25 *harveyi*]. Thus, luciferase alpha and beta subunit-encoding DNA is provided and can be used to produce the luciferase. DNA encoding the  $\alpha$  [1065 bp] and  $\beta$  [984 bp] subunits, DNA encoding a luciferase gene of 2124 bp, encoding the alpha and beta subunits, a recombinant vector containing DNA encoding both subunits and a transformed E. coli and other bacterial hosts for expression and

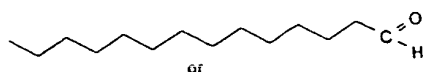
-71-

production of the encoded luciferase are available. In addition, bacterial luciferases are commercially available.

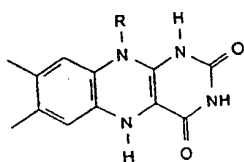
## (2) Luciferins

Bacterial luciferins include:

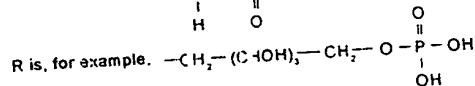
5



10



15



in which the tetradecanal with reduced flavin mononucleotide are considered luciferin since both are oxidized during the light emitting reaction.

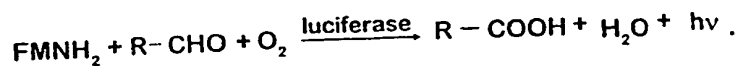
## (3) Reactions

The bacterial systems require, in addition to reduced flavin, five polypeptides to complete the bioluminescent reaction: two subunits,  $\alpha$  and  $\beta$ , of bacterial luciferin and three units of a fatty acid reductase system complex, which supplies the tetradecanal aldehyde. Examples of bacterial bioluminescent systems useful in the apparatus and methods provided herein include those derived from *Vibrio fischeri* and *Vibrio harveyi*. One advantage to this system is its ability to operate at cold temperatures; certain surgical procedures are performed by cooling the body to lower temperatures.

30

Bacterial luciferase catalyzes the flavin-mediated hydroxylation of a long-chain aldehyde to yield carboxylic acid and an excited flavin; the flavin decays to ground state with the concomitant emission of blue green light [ $\lambda_{\text{max}} = 490 \text{ nm}$ ; see, e.g., Legocki *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 81:9080; see U.S. Patent No. 5,196,524]:

-72-



The reaction can be initiated by contacting reduced flavin mononucleotide [FMNH<sub>2</sub>] with a mixture of the bacterial luciferase, oxygen, and a long-chain aldehyde, usually n-decyl aldehyde.

- DNA encoding luciferase from the fluorescent bacterium *Alteromonas* *5 hanedai* is known [CHISSO CORP; see, also, Japanese application JP 7222590, published August 22, 1995]. The reduced flavin mononucleotide [FMNH<sub>2</sub>; luciferin] reacts with oxygen in the presence of bacterial luciferase to produce an intermediate peroxy flavin. This intermediate reacts with a long-chain aldehyde [tetradecanal] to form the acid and the luciferase-bound hydroxy flavin   
 10 in its excited state. The excited luciferase-bound hydroxy flavin then emits light and dissociates from the luciferase as the oxidized flavin mononucleotide [FMN] and water. In vivo FMN is reduced again and recycled, and the aldehyde is regenerated from the acid.

- Flavin reductases have been cloned [see, e.g., U.S. Patent No.   
 15 5,484,723; see, SEQ ID No. 14 for a representative sequence from this patent]. These as well as NAD(P)H can be included in the reaction to regenerate FMNH<sub>2</sub> for reaction with the bacterial luciferase and long chain aldehyde. The flavin reductase catalyzes the reaction of FMN, which is the luciferase reaction, into FMNH<sub>2</sub>; thus, if luciferase and the reductase are included in the reaction   
 20 system, it is possible to maintain the bioluminescent reaction. Namely, since the bacterial luciferase turns over many times, bioluminescence continues as long as a long chain aldehyde is present in the reaction system.

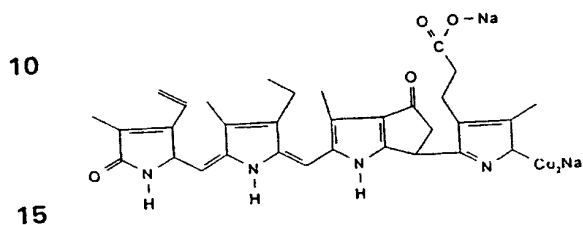
- The color of light produced by bioluminescent bacteria also results from the participation of a protein blue-fluorescent protein [BFP] in the   
 25 bioluminescence reaction. This protein, which is well known [see, e.g., Lee et al. (1978) Methods in Enzymology LVII:226-234], may also be added to bacterial bioluminescence reactions in order to cause a shift in the color.

-73-

## f. Other systems

## (1) Dinoflagellate bioluminescence generating systems

In dinoflagellates, bioluminescence occurs in organelles termed scintillons. These organelles are outpocketings of the cytoplasm into the cell vacuole. The scintillons contain only dinoflagellate luciferase and luciferin (with its binding protein), other cytoplasmic components being somehow excluded. The dinoflagellate luciferin is a tetrapyrrole related to chlorophyll:



or an analog thereof.

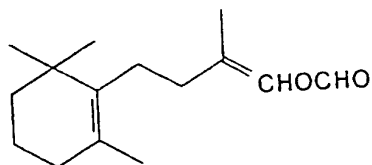
The luciferase is a 135 kD single chain protein that is active at pH 6.5, but inactive at pH 8 [see, e.g., Hastings (1981) Bioluminescence and Chemiluminescence, DeLuca et al., eds. Academic Press, NY, pp.343-360]. Luminescent activity can be obtained in extracts made at pH 8 by simply shifting the pH from 8 to 6. This occurs in soluble and particulate fractions. Within the intact scintillon, the luminescent flash occurs for ~100 msec, which is the duration of the flash *in vivo*. In solution, the kinetics are dependent on dilution, as in any enzymatic reaction. At pH 8, the luciferin is bound to a protein [luciferin binding protein] that prevents reaction of the luciferin with the luciferase. At pH 6, however, the luciferin is released and free to react with the enzyme.

(2) Systems from molluscs, such as *Latia* and *Pholas*

Molluscs *Latia neritoides* and species of *Pholas* are bioluminescent animals. The luciferin has the structure:

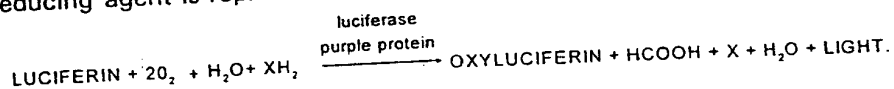
35

-74-



and has been synthesized [see, e.g., Shimomura *et al.* (1968) *Biochemistry* 7:1734-1738; Shimomura *et al.* (1972) *Proc. Natl. Acad. Sci. U.S.A.* 69:2086-2089].

5 In addition to a luciferase and luciferin the reaction has a third component, a "purple protein". The reaction, which can be initiated by an exogenous reducing agent is represented by the following scheme:

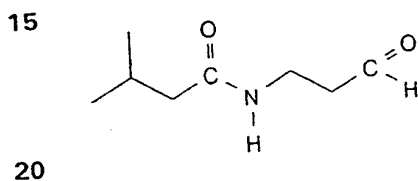


XH<sub>2</sub> is a reducing agent.

10 Thus for practice herein, the reaction will require the purple protein as well as a reducing agent.

### (3) Earthworms and other annelids

Earthworm species, such as *Diplocardia longa*, *Chaetopterus* and *Harmothoe*, exhibit bioluminescence. The luciferin has the structure:



25

The reaction requires hydrogen peroxide in addition to luciferin and luciferase. The luciferase is a photoprotein.

### (4) Glow worms

30 The luciferase/luciferin system from the glow worms that are found in Great Britain, and in Australian and New Zealand caves are also intended for use herein.



-75-

(5) Marine polychaete worm systems

Marine polychaete worm bioluminescence generating systems, such as *Phyxotrix* and *Chaetopterus*, are also contemplated for use herein.

(6) South American railway beetle

5 The bioluminescence generating system from the South American railway beetle is also intended for use herein.

(7) Fish

Of interest herein, are luciferases and bioluminescence generating systems that generate red light. These include luciferases found in species of  
10 *Aristostomias*, such as *A. scintillans* [see, e.g., O'Day *et al.* (1974) *Vision Res.* 14:545-550], *Pachystomias*, *Malacosteus*, such as *M. niger*.

Blue/green emitters include cyclthone, myctophids, hatchet fish (*agyropelecus*), *vinciguerria*, *howella*, *florenciella*, and *Chauliodus*.

g. Fluorescent Proteins

15 The GFP from *Aequorea* and that of the sea pansy *Renilla reniformis* share the same chromophore, yet *Aequorea* GFP has two absorbance peaks at 395 and 475 nm, whereas *Renilla* GFP has only a single absorbance peak at 498 nm, with about 5.5 fold greater monomer extinction coefficient than the major 395 nm peak of the *Aequorea* protein [Ward, W. W. in *Bioluminescence*  
20 and *Chemiluminescence* (eds. DeLuca, M. A. & McElroy, W. D.) 235-242 (Academic Press, New York, 1981)]. The spectra of the isolated chromophore and denatured protein at neutral pH do not match the spectra of either native protein [Cody, C. W. *et al.* (1993) *Biochemistry* 32:1212-1218].

(1) Green and blue fluorescent proteins

25 As described herein, blue light is produced using the *Renilla* luciferase or the *Aequorea* photoprotein in the presence of  $Ca^{2+}$  and the coelenterazine luciferin or analog thereof. This light can be converted into a green light if a green fluorescent protein (GFP) is added to the reaction. Green fluorescent proteins, which have been purified [see, e.g., Prasher *et al.* (1992) *Gene*  
30 111:229-233] and also cloned [see, e.g., International PCT Application No. WO 95/07463, which is based on U.S. application Serial No. 08/119,678 and U.S. application Serial No. 08/192,274, which are herein incorporated by reference],

-76-

are used by cnidarians as energy-transfer acceptors. GFPs fluoresce in vivo upon receiving energy from a luciferase-oxyluciferin excited-state complex or a  $\text{Ca}^{2+}$ -activated photoprotein. The chromophore is modified amino acid residues within the polypeptide. The best characterized GFPs are those of *Aequorea* and

5 *Renilla* [see, e.g., Prasher et al. (1992) Gene 111:229-233; Hart, et al. (1979) Biochemistry 18:2204-2210]. For example, a green fluorescent protein [GFP] from *Aequorea victoria* contains 238 amino acids, absorbs blue light and emits green light. Thus, inclusion of this protein in a composition containing the

10 aequorin photoprotein charged with coelenterazine and oxygen, can, in the presence of calcium, result in the production of green light. Thus, it is contemplated that GFPs may be included in the bioluminescence generating reactions that employ the aequorin or *Renilla* luciferases or other suitable luciferase in order to enhance or alter color of the resulting bioluminescence.

GFPs are activated by blue light to emit green light and thus may be used

15 in the absence of luciferase and in conjunction with an external light source with novelty items, as described herein. Similarly, blue fluorescent proteins (BFPs), such as from *Vibrio fischeri*, *Vibrio harveyi* or *Photobacterium phosphoreum*, may be used in conjunction with an external light source of appropriate wavelength to generate blue light. (See for example, Karatani, et

20 al., "A blue fluorescent protein from a yellow-emitting luminous bacterium," Photochem. Photobiol. 55(2):293-299 (1992); Lee, et al., "Purification of a blue-fluorescent protein from the bioluminescent bacterium *Photobacterium phosphoreum*" Methods Enzymol. (Biolumin. Chemilumin.) 57:226-234 (1978); and Gast, et al. "Separation of a blue fluorescence protein from bacterial

25 luciferase" Biochem. Biophys. Res. Commun. 80(1):14-21 (1978), each, as all references cited herein, incorporated in its entirety by reference herein.) In particular, GFPs, and/or BFPs or other such fluorescent proteins may be used in the beverage and/or food combinations provided herein and served in rooms

30 illuminated with light of an appropriate wavelength to cause the fluorescent proteins to fluoresce.

GFPs and/or BFPs or other such fluorescent proteins may be used in any of the novelty items and combinations provided herein, such as the beverages

-77-

and toys, including bubble making toys, particularly bubble-making compositions or mixtures. Also of particular interest are the use of these proteins in cosmetics, particularly face paints or make-up, hair colorants or hair conditioners, mousses or other such products. Such systems are particularly of interest because no luciferase is needed to activate the photoprotein and because the proteins are non-toxic and safe to apply to the skin, hair, eyes and to ingest. These fluorescent proteins may also be used in addition to bioluminescence generating systems to enhance or create an array of different colors.

These proteins may be used alone or in combination with bioluminescence generating systems to produce an array of colors. They may be used in combinations such that the color of, for example, a beverage changes over time, or includes layers of different colors.

#### (2) Phycobiliproteins

- Phycobiliproteins are water soluble fluorescent proteins derived from cyanobacteria and eukaryotic algae [see, *e.g.*, Apt *et al.* (1995) *J. Mol. Biol.* 238:79-96; Glazer (1982) *Ann. Rev. Microbiol.* 36:173-198; and Fairchild *et al.* (1994) *J. of Biol. Chem.* 269:8686-8694]. These proteins have been used as fluorescent labels in immunoassay [see, Kronick (1986) *J. of Immunolog. Meth.* 92:1-13], the proteins have been isolated and DNA encoding them is also available [see, *e.g.*, Pilot *et al.* (1984) *Proc. Natl. Acad. Sci. U.S.A.* 81:6983-6987; Lui *et al.* (1993) *Plant Physiol* 103:293-294; and Houmard *et al.* (1988) *J. Bacteriol.* 170:5512-5521; the proteins are commercially available from, for example, ProZyme, Inc., San Leandro, CA].
- In these organisms, the phycobiliproteins are arranged in subcellular structures termed phycobilisomes, and function as accessory pigments that participate in photosynthetic reactions by absorbing visible light and transferring the derived energy to chlorophyll via a direct fluorescence energy transfer mechanism.
- Two classes of phycobiliproteins are known based on their color: phycoerythrins (red) and phycocyanins (blue), which have reported absorption maxima between 490 and 570 nm and between 610 and 665 nm, respectively.

-78-

Phycoerythrins and phycocyanins are heterogenous complexes composed of different ratios of alpha and beta monomers to which one or more class of linear tetrapyrrole chromophores are covalently bound. Particular phycobiliproteins may also contain a third  $\gamma$ -subunit which often associated with  $(\alpha\beta)_6$  aggregate proteins.

5 All phycobiliproteins contain either phycothrombilin or phycoerythrobilin chromophores, and may also contain other bilins phycoourobilin, cryptoviolin or the 697 nm bilin. The  $\gamma$ -subunit is covalently bound with phycoourobilin which results in the 495-500 nm absorbtion peak of B- and R-phycoerythrins. Thus, 10 the spectral characteristics of phycobilproetins may be influenced by the combination of the different chromophores, the subunit composition of the apo-phycobiliproteins and/or the local environment effecting the tertiary and quaternary structure of the phycobiliproteins.

As described above for GFPs and BFPs, phycobiliproteins are also 15 activated by visible light of the appropriate wavelength and, thus, may be used in the absence of luciferase and in conjunction with an external light source to illuminate neoplaisa and specialty tissues, as described herein. Furthermore, the attachment of phycobiliproteins to solid support matrices is known (e.g., see U.S. Patent Nos. 4,714,682; 4,767,206; 4,774,189 and 4,867,908). As noted 20 above, these proteins may be used in combination with other fluorescent proteins and/or bioluminescence generating systems to produce an array of colors or to provide different colors over time.

As described above, attachment of phycobiliproteins to solid support matrices is known (e.g., see U.S. Patent Nos. 4,714,682; 4,767,206; 25 4,774,189 and 4,867,908). Therefore, phycobiliproteins may be coupled to microcarriers coupled to one or more components of the bioluminescent reaction, preferably a luciferase, to convert the wavelength of the light generated from the bioluminescent reaction. Microcarriers coupled to one or more phycobiliproteins may be used in any of the methods provided herein. 30 The conversion of blue or green light to light of a longer wavelength, i.e., red or near infra-red, is particularly preferred for the visualization of deep

-79-

neoplasias or specialty tissues using a laparoscope or computer tomogram imaging system, as described herein.

Thus, when a change in the frequency of emitted light is desired, the phycobiliprotein can be included with the bioluminescent generating

5 components.

**C. ISOLATION AND IDENTIFICATION OF NUCLEIC ACIDS ENCODING LUCIFERASES AND GFPs**

Nucleic acid bioluminescent proteins, including two new green fluorescent proteins (GFPs) and three coelenterazine-using luciferases are provided. An advantage of the coelenterazine-using luciferases in many 10 applications, particularly analytical applications, is that only the light-emitting luciferin and molecular oxygen are needed; cofactors such as ATP or  $Ca^{++}$  are not required.

The nucleic acids that encode these luciferases and GFPs can also be used to isolated related nucleic acid from related speices. Also provided herein, 15 are methods for isolating additional genes encoding luciferases and, particularly GFPs, from related species that have heretofore proven difficult to isolate.

Nucleic acids encoding luciferases from *Renilla mulleri*, *Pleuromamma*, *Gaussia* and *Ptilosarcus* have been isolated. These nucleic acids have been or 20 can be introduced into plasmids and expression vectors and into suitable host cells. The host cells have been and can be used to produce the encoded protein, which can be used for any of the applications described herein or known to those of skill in the art.

The cloned DNA fragments can be replicated in bacterial cells, preferably 25 in *E. coli*. A preferred DNA fragment also includes a bacterial origin of replication, to ensure the maintenance of the DNA fragment from generation to generation of the bacteria. In this way, large quantities of the DNA fragment can be produced by replication in bacteria. Preferred bacterial origins of replication include, but are not limited to, the f1-ori and col E1 origins of 30 replication. Preferred hosts contain chromosomal copies of DNA encoding T7 RNA polymerase operably linked to an inducible promoter, such as the lacUV promoter (see, U.S. Patent No. 4,952,496). Such hosts include, but are not

-80-

limited to, lysogens *E. coli* strains HMS174(DE3)pLysS, BL21(DE3)pLysS, HMS174(DE3) and BL21(DE3). Strain BL21(DE3) is preferred. The pLys strains provide low levels of T7 lysozyme, a natural inhibitor of T7 RNA polymerase.

- 5 For expression and for preparation of mutueins, such as temperature sensitive muteins, eukaryotic cells, among them, yeast cells, such as *Saccharomyces* are preferred.

Fusion proteins of the luciferases and GFPs are also provided. Methods of use thereof are also provided.

- 10 The methods are described with respect to *Renilla* and *Gaussia* nucleic acids and proteins. Similar methods were used to identify and isolated the *Ptilosarcus* and *Pleuromamma* nucleic acids and proteins provided herein.

- The GFP cloned from *Renilla mullerei* has spectral properties that make it extremely useful. These properties include very high quantum efficiency, high molar absorbency and efficient use with universally available fluorescein filters (e.g., Endo GFP filter set sold by Chroma). It is known that *Renilla reniformis* GFP is sixfold brighter than the wild-type *Aequorea* GFP on a molar basis, and three to fourfold brighter than the brightest mutant. The *Renilla mulerei* GFP encoded by the nucleic acid clones provided herein exhibits similar functional characteristics, and the spectra appear identical with those from native *reniformis* GFP.
- 15  
20

Based on the excitation and emission curve shapes, the *Ptilosarcus* GFP provided herein has a molar absorbance even higher than that of the *R. mullerei* GFP and should be even brighter.

- 25 The *Guassia* and *Pleuromamma* luciferases are the first two copepod luciferases to be cloned; both are excreted, and so should effective markers for secreted proteins. The *Guassia* luciferase is the smallest luciferase so far found (MW 19,900). All the luciferases show the typical output spectrum of coelenterazine-using luciferases. All show a strong dependance on cation concentration, but do not require divalent cations (data not shown). None of the luciferases has any significant homology with the luciferases isolated from another species.
- 30

-81-

There, however, is considerable homology between the *Ptilosarcus* GFP and the *R. mulleri* GFP (~80%) but little homology with the *A. victoria* GFP (~25%). In spite of this, all three proteins are 238AA in length, suggesting that the structures of all three proteins are similar. Sequence comparison among the GFPs isolated from *Aequorea victoria*, *Renilla mullerei*, and *Ptilosarcus* reveal that the chromophore sequences of *R. mullerei* and *Ptilosarcus* are identical, and differ from *A. victoria*. These sequence differences point to protein sites that can be modified without affecting the essential fluorescence properties and also provide a means to identify residues that change these properties.

#### ISOLATION AND IDENTIFICATION OF NUCLEIC ACID ENCODING *Gaussia* LUCIFERASE

##### 1. Isolation of specimens of the genus *Gaussia*

Specimens of *Gaussia* are readily available from the oceans of the world, including the Gulf of Mexico, Pacific Ocean and Atlantic Ocean. The species used herein for isolation of the exemplified nucleic acid were isolated from the Pacific Ocean off of the Southern California coast in the San Pedro and San Clemente basins. The creatures are identified by sifting through samples of ocean water in the dark and selecting the glowing copepods. Upon capture, the specimens are washed thoroughly and may also be dissected to enrich for light-emitting tissues. The whole organisms or dissected tissues are then snap frozen and stored in liquid nitrogen.

As described in detail in the examples below, whole *Gaussia* were used as a source for isolation of nucleic acids encoding *Gaussia* luciferase (e.g., see SEQ ID No. 19).

##### 2. Preparation of *Gaussia* cDNA expression libraries

*Gaussia* cDNA expression libraries may be prepared from intact RNA following the methods described herein or by other methods known to those of skill in the art (e.g., see Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.; U.S. Patent No. 5,292,658).

-82-

Typically, the preparation of cDNA libraries includes the isolation of polyadenylated RNA from the selected organism followed by single-strand DNA synthesis using reverse transcriptase, digestion of the RNA strand of the DNA/RNA hybrid and subsequent conversion of the single-stranded DNA to double stranded cDNA.

a. RNA isolation and cDNA synthesis

Whole *Gaussia* was used as source of total cytoplasmic RNA for the preparation of *Gaussia* cDNA. Total intact RNA can be isolated using standard techniques well known to those of skill in the art (e.g., see Sambrook *et al.* (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). After isolating total cellular RNA, polyadenylated RNA species are then easily separated from the nonpolyadenylated species using affinity chromatography on oligodeoxythymidylate cellulose columns, (e.g., as described by Aviv *et al.*, (1972) Proc. Natl. Acad. Sci. U.S.A. 69:1408).

The purified *Gaussia* polyA-mRNA is then subjected to a cDNA synthesis reaction to generate a cDNA library from total polyA-mRNA. Briefly, reverse transcriptase is used to extend an annealed polydT primer to generate an RNA/DNA duplex. The RNA strand is then digested using an RNase, e.g., RNase H, and following second-strand synthesis, the cDNA molecules are blunted-ended with S1 nuclease or other appropriate nuclease. The resulting double-stranded cDNA fragments can be ligated directly into a suitable expression vector or, alternatively, oligonucleotide linkers encoding restriction endonuclease sites can be ligated to the 5'-ends of the cDNA molecules to facilitate cloning of the cDNA fragments.

b. Construction of cDNA expression libraries

The best characterized vectors for the construction of cDNA expression libraries are lambda vectors. Lambda-based vectors tolerate cDNA inserts of about 12 kb and provide greater ease in library screening, amplification and storage compared to standard plasmid vectors. Presently preferred vectors for the preparation of *Gaussia* cDNA (and the other libraries herein) expression libraries are the Lambda, Uni-Zap, Lambda-Zap II or Lambda-ZAP



-83-

Express/EcoRI/XhoI vectors, which are known to those of skill in the art (e.g., see U.S. Pat. No. 5,128,256), and are also commercially available (Stratagene, La Jolla, CA).

Generally, the Lambda-Zap vectors combine the high efficiency of a  
5 bacteriophage lambda vector systems with the versatility of a plasmid system. Fragments cloned into these vectors can be automatically excised using a helper phage and recircularized to generate subclones in the pBK-derived phagemid. The pBK phagemid carries the ampicillin -resistance gene (AMP<sup>R</sup>) for selection in bacteria and G418 selection in eukaryotic cells or may contain the  $\beta$ -lactamase  
10 resistance gene. Expression of the recombinant polypeptide is under the control of the *lacZ* promoter in bacteria and the CMV promoter in eukaryotes.

More specifically, these lambda-based vectors are composed of an initiator-terminator cassette containing the plasmid system, e.g., a the well known pBK Bluescript derivative (available from Stratagene), bracketed by the  
15 right and left arm of the bacteriophage lambda. The lambda arms allow for efficient packaging of replicated DNA whereas the excisable initiator-terminator cassette allows for easy cloning of the cDNA fragments and the generation of a plasmid library without the need for additional subcloning.

When used herein, cDNA fragments are inserted into the multiple cloning  
20 site contained within the initiator-terminator cassette of the Lambda-Zap vector to create a set of cDNA expression vectors. The set of cDNA expression vectors is allowed to infect suitable *E. coli* cells, followed by co-infection with a filamentous helper phage. Within the cell, trans-acting proteins encoded by the helper phage, e.g., the gene II protein of M13, recognize two separate domains  
25 positioned within the lambda arms of the vector and introduce single-stranded nicks flanking the initiator-terminator cassette. Upon a subsequent round of DNA synthesis, a new DNA strand is synthesized that displaces the existing nick strand liberating the initiator-terminator cassette. The displaced strand is then circularized, packaged as filamentous phage by the helper proteins and  
30 excreted from the cell. The BK plasmid containing the cDNA is recovered by infecting an F' strain of *E. coli* and plating the infected cells on solid medium supplemented with ampicillin for the selection of pBK-containing cells.

-84-

The *Gaussia* cDNA expression library can be screened using a variety of methods known to those of skill in the art. For example, identification of *Gaussia* luciferase may be achieved using a functional screening method by observing colonies visually for emission of blue light or by observing light emission using one or more bandpass filter.

### 3. Isolation and identification of DNA encoding *Gaussia* luciferase

DNA encoding a *Gaussia* luciferase may be isolated using methods described herein, or by using other methods known to those of skill in the art. As described in detail below, a *Gaussia*  $\lambda$  Uni-Zap cDNA expression plasmid library was prepared, transformed into competent *E. coli* cells and plated onto modified L-broth plates containing carbon black to absorb background fluorescence (e.g., see EXAMPLES).

Transformants were sprayed with a solution containing IPTG (isopropyl  $\beta$ -D-thiogalactopyranoside; see, et al. Nakamura et al. (1979) Cell 18:1109-1117) to induce expression of the recombinant *Gaussia* luciferase from the heterologous DNA. Other induction systems may also be used. Preferred promoter regions are those that are inducible and functional in *E. coli* or early genes in vectors of viral origin. Examples of suitable inducible promoters and promoter regions include, but are not limited to: the *E. coli* lac operator responsive to isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG; see, et al. Nakamura et al. (1979) Cell 18:1109-1117); the metallothionein promoter metal-regulatory-elements responsive to heavy-metal (e.g., zinc) induction (see, e.g., U.S. Patent No. 4,870,009 to Evans et al.); the phage T7lac promoter responsive to IPTG (see, e.g., U.S. Patent No. 4,952,496; and Studier et al. (1990) Meth. Enzymol. 185:60-89) and the TAC promoter. Other promoters include, but are not limited to, the T7 phage promoter and other T7-like phage promoters, such as the T3, T5 and SP6 promoters, the trp, lpp, and lac promoters, such as the lacUV5, from *E. coli*; the P10 or polyhedrin gene promoter of baculovirus/insect cell expression systems (see, e.g., U.S. Patent Nos. 5,243,041, 5,242,687, 5,266,317, 4,745,051, and 5,169,784) and inducible promoters from other eukaryotic expression systems.

-85-

Particularly preferred plasmids for transformation of *E. coli* cells include the pET expression vectors (see, U.S. patent 4,952,496; available from NOVAGEN, Madison, WI; see, also literature published by Novagen describing the system). Such plasmids include, pET 34 (see Fig. 1), pET 11a, which  
5 contains the T7lac promoter, T7 terminator, the inducible *E. coli* lac operator, and the lac repressor gene; pET 12a-c, which contains the T7 promoter, T7 terminator, and the *E. coli* ompT secretion signal; and pET 15b (NOVAGEN, Madison, WI), which contains a His-Tag™ leader sequence) for use in purification with a His column and a thrombin cleavage site that permits  
10 cleavage following purification over the column; the T7-lac promoter region and the T7 terminator. Plasmid pET 34 further includes the CBD to aid in purification.

Particularly preferred plasmids for transformation of *E. coli* cells include the pET expression vectors (see, U.S. patent 4,952,496; available from  
15 NOVAGEN, Madison, WI). For example, the plasmid pET34-LIC is a prokaryotic expression vector that contains a multiple cloning site for inserting heterologous DNA templates downstream from a bacteriophage T7 promoter. Transformation into a bacterial host that expresses T7 RNA polymerase, e.g., *E. coli* strain BL21(DE3), results in high level, recombinant expression of the heterologous  
20 protein. DNA encoding the *Gaussia* luciferase has been inserted into the pET34 vector as a fusion with the cellulose binding domain (CBD; see, SEQ ID Nos. 21 and 22), and expressed in *E. coli* host cells.

Over 120 different CBD sequences have been identified and grouped into at least 10 families on the basis of sequence similarities (Tomme *et al.* (1995)  
25 in *Enzymatic Degradation of Insoluble Polysaccharides*; Saddler, J. M., and Penner, M., Eds.; American Chemical Society, Washington, D.C; pp 142-161). The CBD<sub>cl</sub>-Tag sequence is derived from the Cellulose-Binding Protein A (CbpA) of  
*Clostridium cellulovorans* (Goldstein *et al.* (1993) *J. Bacteriol.* 175:5762-5768)  
30 and has a high affinity for crystalline cellulose.

To identify luciferase-expressing clones, transformants, grown on black agar, were sprayed with coelenterazine.

-86-

Expression was apparent because of the resulting colonies that emit an intense blue-green light. Glowing colonies were selected. The nucleotide sequence of the cDNA insert of a blue light-emitting transformant was determined (e.g., see SEQ ID No. 19). As described in herein the 765 DNA insert encodes a 185 amino acid polypeptide.

### ISOLATION AND IDENTIFICATION OF NUCLEIC ACID ENCODING *Renilla* PROTEINS

#### 1. Isolation of specimens of the genus *Renilla*

Specimens of *Renilla* are readily available from the oceans of the world, including the Gulf of Mexico, Pacific Ocean and Atlantic Ocean. *Renilla* typically live on the ocean bottom at about 30 to 100 feet deep and can be easily collected by dragging. For example, specimens of *R. kollikeri* can be obtained off the coast of California or Baja, Mexico. Alternatively, live specimens of *Renilla* may be purchased from a commercial supplier (e.g., Gulf Marine Incorporated, Panacea, Fla). Upon capture or receipt, the specimens are washed thoroughly and may also be dissected to enrich for light-emitting tissues. The whole organisms or dissected tissues are then snap frozen and stored in liquid nitrogen.

As described in detail in the examples below, the frozen tissues were used as a source to isolate nucleic acids encoding *Renilla mulleri* GFP and luciferase (e.g., see SEQ ID No. 15 and SEQ ID No. 17, respectively).

#### 2. Preparation of *Renilla* cDNA expression libraries

*Renilla* cDNA expression libraries may be prepared from intact RNA following the methods described herein or by other methods known to those of skill the art (e.g., see Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.; U.S. Patent No. 5,292,658).

Typically, the preparation of cDNA libraries includes the isolation of polyadenylated RNA from the selected organism followed by single-strand DNA synthesis using reverse transcriptase, digestion of the RNA strand of the DNA/RNA hybrid and subsequent conversion of the single-stranded DNA to double stranded cDNA.

-87-

a. RNA isolation and cDNA synthesis

Whole *Renilla* or dissected *Renilla* tissues can be used a source of total cytoplasmic RNA for the preparation of *Renilla* cDNA. Total intact RNA can be isolated from crushed *Renilla* tissue, for example, by using a modification of methods generally known in the art (e.g., see Chirgwin *et al.* (1970) Biochemistry 18:5294-5299). After isolating total cellular RNA, polyadenylated RNA species are then easily separated from the nonpolyadenylated species using affinity chromatography on oligodeoxythymidylate cellulose columns, (e.g., as described by Aviv *et al.*, (1972) Proc. Natl. Acad. Sci. U.S.A. 69:1408).

The purified *Renilla* polyA-mRNA is then subjected to a cDNA synthesis reaction to generate a cDNA library from total polyA-mRNA. Briefly, reverse transcriptase is used to extend an annealed polydT primer to generate an RNA/DNA duplex. The RNA strand is then digested using an RNase, e.g., RNase H, and following second-strand synthesis, the cDNA molecules are blunted-ended with S1 nuclease or other appropriate nuclease. The resulting double-stranded cDNA fragments can be ligated directly into a suitable expression vector or, alternatively, oligonucleotide linkers encoding restriction endonuclease sites can be ligated to the 5'-ends of the cDNA molecules to facilitate cloning of the cDNA fragments.

b. Construction of cDNA expression libraries

The best characterized vectors for the construction of cDNA expression libraries are lambda vectors. Lambda-based vectors tolerate cDNA inserts of about 12 kb and provide greater ease in library screening, amplification and storage compared to standard plasmid vectors. Presently preferred vectors for the preparation of *Renilla* cDNA expression libraries are the Lambda, Uni-Zap, Lambda-Zap II or Lambda-ZAP Express/EcoRI/XhoI vectors, which are known to those of skill in the art (e.g., see U.S. Pat. No. 5,128,256), and are also commercially available (Stratagene, La Jolla, CA).

Generally, the Lambda-Zap vectors combine the high efficiency of a bacteriophage lambda vector systems with the versatility of a plasmid system. Fragments cloned into these vectors can be automatically excised using a helper

-88-

phage and recircularized to generate subclones in the pBK-derived phagemid. The pBK phagemid carries the neomycin-resistance gene for selection in bacteria and G418 selection in eukaryotic cells or may contain the  $\beta$ -lactamase resistance gene. Expression of the recombinant polypeptide is under the control of the *lacZ* promoter in bacteria and the CMV promoter in eukaryotes.

More specifically, these lambda-based vectors are composed of an initiator-terminator cassette containing the plasmid system, e.g., a pBK Bluescript derivative (Stratagene, San Diego), bracketed by the right and left arm of the bacteriophage lambda. The lambda arms allow for efficient packaging of replicated DNA whereas the excisable initiator-terminator cassette allows for easy cloning of the cDNA fragments and the generation of a plasmid library without the need for additional subcloning.

When used herein, cDNA fragments are inserted into the multiple cloning site contained within the initiator-terminator cassette of the Lambda-Zap vector to create a set of cDNA expression vectors. The set of cDNA expression vectors is allowed to infect suitable *E. coli* cells, followed by co-infection with a filamentous helper phage. Within the cell, trans-acting proteins encoded by the helper phage, e.g., the gene II protein of M13, recognize two separate domains positioned within the lambda arms of the vector and introduce single-stranded nicks flanking the initiator-terminator cassette. Upon a subsequent round of DNA synthesis, a new DNA strand is synthesized that displaces the existing nick strand liberating the initiator-terminator cassette. The displaced strand is then circularized, packaged as filamentous phage by the helper proteins and excreted from the cell. The BK plasmid containing the cDNA is recovered by infecting an F' strain of *E. coli* and plating the infected cells on solid medium supplemented with kanamycin for the selection of pBK-containing cells.

The *Renilla* cDNA expression library can be screened using a variety of methods known to those of skill in the art. For example, identification of *Renilla* GFP may be achieved using a functional screening method employing blue light and observing colonies visually for emission of green fluorescence or by observing light emission using one or more bandpass filter.

### 3. Isolation and identification of DNA encoding *Renilla* GFP

-89-

DNA encoding a *Renilla* GFP may be isolated using methods described herein, or by using other methods known to those of skill in the art. As described in detail below, a *R. mulleri*  $\lambda$  Uni-Zap cDNA expression plasmid library was prepared, transformed into competent *E. coli* cells and plated onto modified L-broth plates containing carbon black to absorb background fluorescence (e.g., see EXAMPLE 4). Transformants were sprayed with a solution containing IPTG to induce expression of the recombinant *Renilla* GFP from the heterologous cDNA. To identify GFP expressing clones, transformants were placed in blue light, preferably 470 to 490 nm light, and colonies that emitted green fluorescence were isolated and grown in pure culture.

The nucleotide sequence of the cDNA insert of a green fluorescent transformant was determined (e.g., see SEQ ID No. 15). As described in EXAMPLE 4, the 1,079 cDNA insert encodes a 238 amino acid polypeptide that is only 23.5 % identical to *A. victoria* GFP, the only other GFP that has been characterized at the molecular level. The recombinant protein exhibits excitation and emission spectra similar to those reported for live *Renilla* species.

#### 4. Isolation and identification of DNA encoding *Renilla* luciferase

The above-described *R. mulleri* cDNA expression library was also used to clone DNA encoding a *R. mulleri* luciferase (e.g., see EXAMPLE 5). Single colony transformants were grown on modified L-broth plates containing carbon black and expression from the heterologous DNA was induced with IPTG, essentially as described above. After allowing time for expression, the transformants were sprayed with coelenterazine and screened for those colonies that emit blue light. Light-emitting colonies were isolated and grown in pure culture.

The nucleotide sequence of the cDNA insert contained in the light-emitting transformant was determined. As described in EXAMPLE 5, the 1,217 cDNA insert encodes a 311 amino acid polypeptide. The recombinant protein exhibits excitation and emission spectra similar to those reported for live *Renilla* species.

-90-

**D. NUCLEIC ACID PROBES AND METHODS FOR ISOLATING AND CLONING OF LUCIFERASE- and GFP-ENCODING NUCLEIC ACIDS FROM OTHER SPECIES**

***Gaussia***

5 The nucleic acid exemplified herein that encodes the *Gaussia* luciferase may be used as a source of probes for isolating luciferases from other *Gaussia* species. Any suitable probe based upon the exemplified sequence of nucleotides may be used in any method. Such probe should hybridize under conditions of at least low stringency, more preferably moderate stringency and  
10 most preferably high stringency to related nucleic acids in a suitable *Gaussia* library.

Also provided herein are specific nucleic acid probes for isolating and cloning luciferase-encoding nucleic acid from other species of *Gaussia*.

Typically the nucleic acid probes are degenerate probes, which are then used  
15 as hybridization probes to screen cDNA libraries prepared from the selected *Gaussia* species to obtain a DNA clone encoding a full-length *Gaussia* luciferase.

Preferred nucleic acid probes are designed to be degenerate probes of at least 14 nucleotides, preferably 16 to 30 nucleotides, that are based on these conserved amino acid positions. For example, particularly preferred regions for  
20 designing probes are based on amino acids 1 to 185, set forth in SEQ ID No. 20. In other preferred embodiments, the nucleic acid probes encoding the above-described preferred amino acid regions are selected among the sequence of nucleotides encoding these regions set forth in SEQ ID NO. 19.

Alternatively, peptides corresponding to these amino acid positions can  
25 be prepared and used as immunogens to immunize animals to produce *Gaussia* luciferase-specific polyclonal or monoclonal antibodies using methods well known to those of skill in the art (e.g., see Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). The antibodies can be used to screen cDNA expression libraries,  
30 such as those prepared following the methods described herein, to identify clones expressing a partial or full-length clones.



-91-

**NUCLEIC ACID PROBES AND METHODS FOR ISOLATING AND  
CLONING OF GFP-ENCODING NUCLEIC ACIDS FROM OTHER  
SPECIES OF *Renilla***

The nucleic acid exemplified herein that encodes the *Renilla mulleri* GFP  
5 may be used as a source of probes for isolating GFPs from other *Renilla* species.  
Any suitable probe based upon the exemplified sequence of nucleotides may be  
used in any method. Such probe should hybridize under conditions of low  
stringency to related nucleic acid in a suitable *Renilla* library.

Also provided herein are specific nucleic acid probes for isolating and  
10 cloning GFP-encoding nucleic acid from other species of *Renilla*. These probes  
are based on regions of the *Renilla* GFP protein that are shared amongst  
members of the *Renilla* genus (see Figure 1). Typically the nucleic acid probes  
are degenerate probes, which are then used as hybridization probes to screen  
cDNA libraries prepared from the selected *Renilla* species to obtain a DNA clone  
15 encoding a full-length *Renilla* GFP.

To elucidate regions of the GFP that are shared amongst of *Renilla*  
species, purified *Renilla reniformis* GFP was subjected to specific chemical and  
proteolytic degradation, e.g., trypsin and Proteinase Q, to produce a variety of  
short peptides for analysis and the amino acid sequence of the *Renilla reniformis*  
20 peptides was determined.

Figure 1 displays an alignment of the deduced amino acid sequence of  
*Renilla mulleri* green fluorescent protein and the amino acid sequence  
determined for the isolated *Renilla reniformis* GFP peptides. Although the two  
species are closely related, the amino acid sequences of the *Renilla* GFPs are  
25 different. This difference, however, can be exploited to construct specific  
probes because there are highly conserved regions. The *R. mulleri* and *R.*  
*reniformis* sequences are identical at 103 of 187 residues present in peptides of  
sufficient length to yield satisfactory alignments.

Certain regions of the two amino acid sequences exhibit a high degree of  
30 conservation. For instance, 18 of 19 amino acids corresponding to positions 51  
to 69 of the *Renilla mulleri* sequence are identical between the two *Renilla*  
GFPs, including a contiguous stretch of 16 identical amino acid residues which

-92-

correspond to amino acid positions 51 to 65. Also, as shown in Figure 1, *Renilla reniformis* GFP (e.g., see SEQ ID No. 20) shares a fairly high degree of sequence similarity with the amino acid residues corresponding to amino acids 81 to 106 of the *R. mulleri* sequence (60.9 %; 18 of 26 identical amino acids).

5 Therefore, these regions provide the sequence for construction of probes.

Preferred nucleic acid probes are designed to be degenerate probes of at least 14 nucleotides, preferably 16 to 30 nucleotides, that are based on these conserved amino acid positions. For example, particularly preferred regions for designing probes are based on amino acids 51 to 68, 82 to 98 and 198 to 208  
10 set forth in SEQ ID No. 16, amino acid sequence set forth in SEQ ID No. 20, amino acids 9-20 set forth in SEQ ID No. 21 and amino acids 39-53 set forth in SEQ ID No. 23. In other embodiments, the nucleic acid probes encoding the above-described preferred amino acid regions are selected among the sequence of nucleotides encoding these regions set forth in SEQ ID NO. 15. These  
15 degenerate nucleic acid probes can be used as hybridization probes for the isolation and cloning of GFP-encoding DNA in *Renilla reniformis* and other species. Alternatively or in addition, these probes may be used as primers in nucleic acid amplification reactions.

Alternatively, peptides corresponding to these amino acid positions can  
20 be prepared and used as immunogens to immunize animals to produce *Renilla* GFP-specific polyclonal or monoclonal antibodies using methods well known to those of skill in the art (e.g., see Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). The antibodies can be used to screen cDNA expression libraries, such as  
25 those prepared following the methods described herein, to identify clones expressing a partial or full-length clone encompassing all or a portion of amino acid residues 51 to 69 of the *Renilla mulleri* GFP (e.g., see Figure 1; SEQ ID Nos. 15 and 16).

-93-

**Other species**

Similar methods may be used with the nucleic acids provided herein that encode the *Ptilosarcus* and *Pleuromamma* proteins.

**E. RECOMBINANT EXPRESSION OF PROTEINS****5      *Gaussia*****1.      DNA encoding *Gaussia* proteins**

As described above, DNA encoding a *Gaussia* luciferase can be isolated from natural sources, synthesized based on *Gaussia* nucleic acid sequences provided herein or prepared using a number of recombinant DNA cloning and  
10      amplification techniques, e.g., polymerase chain reaction (PCR).

In preferred embodiments, the DNA fragment encoding a *Gaussia* luciferase has the sequence of amino acids set forth in SEQ ID No. 20. In more preferred embodiments, the DNA fragment encodes the sequence of amino acids encoded by nucleotides 37-591 of the sequence of nucleotides set forth in  
15      SEQ ID No. 19.

**2.      DNA constructs for recombinant production of *Gaussia* proteins**

DNA is introduced into a plasmid for expression in a desired host. In preferred embodiments, the host is a bacterial host. The sequences of  
20      nucleotides in the plasmids that are regulatory regions, such as promoters and operators, are operationally associated with one another for transcription of the sequence of nucleotides that encode a *Gaussia* luciferase. The sequence of nucleotides encoding the *Gaussia* luciferase may also include DNA encoding a secretion signal, whereby the resulting peptide is a precursor of the *Gaussia*  
25      luciferase

In preferred embodiments the DNA plasmids also include a transcription terminator sequence. The promoter regions and transcription terminators are each independently selected from the same or different genes.

A wide variety of multipurpose vectors suitable for the expression of  
30      heterologous proteins are known to those of skill in the art and are commercially available. Expression vectors containing inducible promoters or constitutive promoters that are linked to regulatory regions are preferred. Such promoters

-94-

include, but are not limited to, the T7 phage promoter and other T7-like phage promoters, such as the T3, T5 and SP6 promoters, the trp, lpp, tet and lac promoters, such as the lacUV5, from E. coli; the SV40 promoter; the P10 or polyhedron gene promoter of baculovirus/insect cell expression systems, 5 retroviral long-terminal repeats and inducible promoters from other eukaryotic expression systems.

### 3. Host organisms for recombinant production of *Gaussia* proteins

Host organisms include those organisms in which recombinant 10 production of heterologous proteins have been carried out, such as, but not limited to, bacteria (for example, E. coli), yeast (for example, Saccharomyces cerevisiae and Pichia pastoris), fungi, baculovirus/insect systems, amphibian cells, mammalian cells, plant cells and insect cells. Presently preferred host organisms are strains of bacteria or yeast. Most preferred host organisms are 15 strains of E. coli or Saccharomyces cerevisiae.

### 4. Methods for recombinant production of *Gaussia* proteins

The DNA encoding a *Gaussia* luciferase is introduced into a plasmid in operative linkage to an appropriate promoter for expression of polypeptides in a selected host organism. The DNA fragment encoding the *Gaussia* luciferase 20 may also include a protein secretion signal that functions in the selected host to direct the mature polypeptide into the periplasm or culture medium. The resulting luciferase can be purified by methods routinely used in the art, including methods described hereinafter in the Examples.

Methods of transforming suitable host cells, preferably bacterial cells, 25 and more preferably E. coli cells, as well as methods applicable for culturing said cells containing a gene encoding a heterologous protein, are generally known in the art. See, for example, Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

30 Once the *Gaussia*-encoding nucleic acid molecule has been introduced into the host cell, the desired protein is produced by subjecting the host cell to conditions under which the promoter is induced, whereby the operatively linked

-95-

DNA is transcribed. The cellular extracts of lysed cells containing the protein may be prepared and the resulting "clarified lysate" employed as a source of the luciferase. Alternatively, the lysate may be subjected to additional purification steps (e.g., ion exchange chromatography or immunoaffinity chromatography) to further enrich the lysate or provide a homogeneous source of the purified enzyme (see e.g., U.S. Patent Nos. 5,292,658 and 5,418,155).

### ***Renilla***

#### **1. DNA encoding *Renilla* proteins**

As described above, DNA encoding a *Renilla* GFP or *Renilla* luciferase can be isolated from natural sources, synthesized based on *Renilla* sequences provided herein or prepared using a number of recombinant DNA cloning and amplification techniques, e.g., polymerase chain reaction (PCR).

In preferred embodiments, the DNA fragment encoding a *Renilla* GFP has the sequence of amino acids set forth in SEQ ID No. 16. In more preferred embodiments, the DNA fragment encodes the sequence of amino acids encoded by nucleotides 259-972 of the sequence of nucleotides set forth in SEQ ID No. 15.

In preferred embodiments, the DNA fragment encoding a *Renilla* luciferase has the sequence of amino acids set forth in SEQ ID No. 18. In more preferred embodiments, the DNA fragment encodes the sequence of amino acids encoded by nucleotides 31-963 of the sequence of nucleotides set forth in SEQ ID No. 17.

#### **2. DNA constructs for recombinant production of *Renilla* proteins**

DNA is introduced into a plasmid for expression in a desired host. In preferred embodiments, the host is a bacterial host. The sequences of nucleotides in the plasmids that are regulatory regions, such as promoters and operators, are operationally associated with one another for transcription of the sequence of nucleotides that encode a *Renilla* GFP or luciferase. The sequence of nucleotides encoding the FGF mutein may also include DNA encoding a secretion signal, whereby the resulting peptide is a precursor of the *Renilla* GFP.

-96-

In preferred embodiments the DNA plasmids also include a transcription terminator sequence. The promoter regions and transcription terminators are each independently selected from the same or different genes.

A wide variety of multipurpose vectors suitable for the expression of heterologous proteins are known to those of skill in the art and are commercially available. Expression vectors containing inducible promoters or constitutive promoters that are linked to regulatory regions are preferred. Such promoters include, but are not limited to, the T7 phage promoter and other T7-like phage promoters, such as the T3, T5 and SP6 promoters, the trp, lpp, tet and lac promoters, such as the lacUV5, from E. coli; the SV40 promoter; the P10 or polyhedron gene promoter of baculovirus/insect cell expression systems, retroviral long-terminal repeats and inducible promoters from other eukaryotic expression systems.

Particularly preferred vectors for recombinant expression of *Renilla mulleri* in prokaryotic organisms are lac- and T7 promoter-based vectors, such as the well known Bluescript vectors, which are commercially available (Stratagene, La Jolla, CA).

### 3. Host organisms for recombinant production of *Renilla* proteins

Host organisms include those organisms in which recombinant production of heterologous proteins have been carried out, such as, but not limited to, bacteria (for example, E. coli), yeast (for example, Saccharomyces cerevisiae and Pichia pastoris), fungi, baculovirus/insect systems, amphibian cells, mammalian cells, plant cells and insect cells. Presently preferred host organisms are strains of bacteria or yeast. Most preferred host organisms are strains of E. coli or Saccharomyces cerevisiae.

### 4. Methods for recombinant production of *Renilla* proteins

The DNA encoding a *Renilla* GFP or *Renilla mulleri* luciferase is introduced into a plasmid in operative linkage to an appropriate promoter for expression of polypeptides in a selected host organism. The DNA fragment encoding the *Renilla* GFP or luciferase may also include a protein secretion signal that functions in the selected host to direct the mature polypeptide into the

-97-

periplasm or culture medium. The resulting *Renilla* GFP or luciferase can be purified by methods routinely used in the art, including methods described hereinafter in the Examples.

Methods of transforming suitable host cells, preferably bacterial cells, and more preferably *E. coli* cells, as well as methods applicable for culturing said cells containing a gene encoding a heterologous protein, are generally known in the art. See, for example, Sambrook *et al.* (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Once the *Renilla*-encoding DNA fragment has been introduced into the host cell, the desired *Renilla* GFP is produced by subjecting the host cell to conditions under which the promoter is induced, whereby the operatively linked DNA is transcribed. The cellular extracts of lysed cells containing the protein may be prepared and the resulting "clarified lysate" was employed as a source of recombinant *Renilla* GFP or *Renilla mulleri* luciferase. Alternatively, the lysate may be subjected to additional purification steps (e.g., ion exchange chromatography or immunoaffinity chromatography) to further enrich the lysate or provide a homogeneous source of the purified enzyme (see *e.g.*, U.S. Patent Nos. 5,292,658 and 5,418,155).

**20 F. RECOMBINANT CELLS EXPRESSING HETEROLOGOUS NUCLEIC ACID ENCODING A LUCIFERASES AND GFPs**

These cells, vectors and methods are exemplified with respect to *Renilla* and *Gaussia*. The same cells, vectors and methods may be used for expressing the *Pleuromamma* and *Ptilosarcus* proteins.

**25 *Gaussia***

Recombinant cells containing heterologous nucleic acid encoding a *Gaussia* luciferase are provided. In preferred embodiments, the recombinant cells express the encoded *Gaussia* luciferase which is functional and non-toxic to the cell. In more preferred embodiments, the *Gaussia* luciferase contains the amino acid sequence set forth in SEQ ID No. 20.

In certain embodiments, the recombinant cells may also include heterologous nucleic acid encoding another component(s) of a bioluminescence-

-98-

generating system, preferably a fluorescent protein. In preferred embodiments, the nucleic acid encoding the bioluminescence-generating system component is isolated from the species *Aequorea*, *Vargula* or *Renilla*. In more preferred embodiments, the additional bioluminescence-generating system component is a

5 *Renilla mulleri* or *reniformis* GFP.

The *Renilla* GFP and GFP peptides can be isolated from natural sources or isolated from a prokaryotic or eukaryotic cell transfected with nucleic acid that encodes the *Renilla* GFP and/or GFP peptides, such as those described above.

10 Exemplary cells include bacteria (e.g., *E. coli*), plant cells, cells of mammalian origin (e.g., COS cells, mouse L cells, Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK) cells, African green monkey cells and other such cells known to those of skill in the art), amphibian cells (e.g., *Xenopus laevis* oocytes), yeast cells (e.g., *Saccharomyces cerevisiae*, *Pichia*  
15 *pastoris*), and the like. Exemplary cells for expressing injected RNA transcripts include *Xenopus laevis* oocytes. Eukaryotic cells that are preferred for transfection of DNA are known to those of skill in the art or may be empirically identified, and include HEK293 (which are available from ATCC under accession #CRL 1573); Ltk cells (which are available from ATCC under accession  
20 #CCL1.3); COS-7 cells (which are available from ATCC under accession #CRL 1651); and DG44 cells (dhfr<sup>-</sup> CHO cells; see, e.g., Urlaub et al. (1986) Cell. Molec. Genet. 12: 555). Presently preferred cells include strains of bacteria and yeast.

The recombinant cells that contain the heterologous DNA encoding the  
25 *Gaussia* luciferase are produced by transfection with DNA encoding a *Gaussia* luciferase or by introduction of RNA transcripts of DNA encoding *Gaussia* proteins using methods well known to those of skill in the art. The DNA may be introduced as a linear DNA fragment or may be included in an expression vector for stable or transient expression of the encoding DNA.

30 Heterologous DNA may be maintained in the cell as an episomal element or may be integrated into chromosomal DNA of the cell. The resulting recombinant cells may then be cultured or subcultured (or passaged, in the case



-99-

of mammalian cells) from such a culture or a subculture thereof. Also, DNA may be stably incorporated into cells or may be transiently expressed using methods known in the art.

The recombinant cells can be used in a wide variety of cell-based assay methods, such as those methods described for cells expressing wild type or modified *A. victoria* GFPs or GFP fusion proteins (e.g., see U.S. Patent No. 5,625,048; International patent application Publication Nos. WO 95/21191; WO 96/23810; WO 96/27675; WO 97/26333; WO 97/28261; WO 97/41228; and WO 98/02571).

10      **RECOMBINANT CELLS EXPRESSING HETEROLOGOUS NUCLEIC ACID  
ENCODING A *Renilla* GREEN FLUORESCENT PROTEIN AND/OR  
LUCIFERASE**

Recombinant cells containing heterologous nucleic acid encoding a *Renilla* GFP are provided. In preferred embodiments, the recombinant cells express the encoded *Renilla* GFP which is functional and non-toxic to the cell.

In certain embodiments, the recombinant cells may also include heterologous nucleic acid encoding a component of a bioluminescence-generating system, preferably a photoprotein or luciferase. In preferred embodiments, the nucleic acid encoding the bioluminescence-generating system component is isolated from the species *Aequorea*, *Vargula* or *Renilla*. In more preferred embodiments, the bioluminescence-generating system component is a *Renilla mulleri* luciferase having the amino acid sequence set forth in SEQ ID No. 18.

Recombinant host cells containing heterologous nucleic acid encoding a *Renilla mulleri* luciferase are also provided. In preferred embodiments, the heterologous nucleic acid encodes the sequence of amino acids as set forth in SEQ ID No. 18. In more preferred embodiments, the heterologous nucleic acid encodes the sequence of nucleotides set forth in SEQ ID No. 17.

Exemplary cells include bacteria (e.g., *E. coli*), plant cells, cells of mammalian origin (e.g., COS cells, mouse L cells, Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK) cells, African green monkey cells and other such cells known to those of skill in the art), amphibian cells (e.g.,

-100-

*Xenopus laevis* oocytes), yeast cells (e.g., *Saccharomyces cerevisiae*, *Pichia pastoris*), and the like. Exemplary cells for expressing injected RNA transcripts include *Xenopus laevis* oocytes. Eukaryotic cells that are preferred for transfection of DNA are known to those of skill in the art or may be empirically identified, and include HEK293 (which are available from ATCC under accession #CRL 1573); Ltk<sup>-</sup> cells (which are available from ATCC under accession #CCL1.3); COS-7 cells (which are available from ATCC under accession #CRL 1651); and DG44 cells (dhfr<sup>-</sup> CHO cells; see, e.g., Urlaub et al. (1986) Cell. Molec. Genet. 12: 555). Presently preferred cells include strains of bacteria and yeast.

The recombinant cells that contain the heterologous DNA encoding the *Renilla* GFP are produced by transfection with DNA encoding a *Renilla* GFP or luciferase or by introduction of RNA transcripts of DNA encoding a *Renilla* proteins using methods well known to those of skill in the art. The DNA may be introduced as a linear DNA fragment or may be included in an expression vector for stable or transient expression of the encoding DNA.

Heterologous DNA may be maintained in the cell as an episomal element or may be integrated into chromosomal DNA of the cell. The resulting recombinant cells may then be cultured or subcultured (or passaged, in the case of mammalian cells) from such a culture or a subculture thereof. Also, DNA may be stably incorporated into cells or may be transiently expressed using methods known in the art.

The recombinant cells can be used in a wide variety of cell-based assay methods, such as those methods described for cells expressing wild type or modified *A. victoria* GFPs or GFP fusion proteins (e.g., see U.S. Patent No. 5,625,048; International patent application Publication Nos. WO 95/21191; WO 96/23810; WO 96/27675; WO 97/26333; WO 97/28261; WO 97/41228; and WO 98/02571).

#### G. Luciferases

Purified *Gaussia* luciferase and *Gaussia* luciferase peptides as wells as *Pleuromamma* and *Renilla mulleri* luciferases are provided. The luciferase is

-101-

produced by expressing the protein in selected host cells and isolating the resulting luciferase.

Nucleic acid encoding a *Renilla mulleri* luciferase is also provided. The nucleic acid is used to produce the encoded luciferase. Presently preferred

5 *Renilla mulleri* luciferase for use in compositions, combinations and methods has the amino acid sequence set forth in SEQ ID No. 18. The luciferase can be formulated for compositions and combinations that have a wide variety of end-use applications, such as those described herein.

#### H. *Renilla* and *Ptilosarcus* GFPs

10 Purified *Renilla* GFPs, particularly *Renilla mulleri* GFP, and purified *Renilla reniformis* GFP peptides are provided. Presently preferred *Renilla* GFP for use in the compositions herein is *Renilla mulleri* GFP having the sequence of amino acids set forth in SEQ ID No. 16. Presently preferred *Renilla reniformis* GFP peptides are those containing the GFP peptides selected from the amino acid

15 sequences set forth in SEQ ID Nos 19-23.

The *Renilla* GFP and GFP peptides can be isolated from natural sources or isolated from a prokaryotic or eukaryotic cell transfected with nucleic acid that encodes the *Renilla* GFP and/or GFP peptides, such as those described in Section F above.

#### 20 I. COMPOSITIONS

As above, compositions and conjugates and methods of use are described with reference to *Gaussia* and *Renilla* proteins and nucleic acids. The same compositions and methods for preparation and use thereof are intended for use with *Pleuromamma* and *Ptilosarcus* proteins and nucleic acids.

##### 25 1. *Gaussia* luciferase compositions

Compositions containing a *Gaussia* luciferase are provided. The compositions may also contain a *Renilla* GFP or GFP peptide. The compositions can take any of a number of forms, depending on the intended method of use therefor. In certain embodiments, the compositions are prepared for use in

30 bioluminescent novelty items, immunoassays or FRET and FET assays. The compositions may also be used in conjunction with multi-well assay devices containing integrated photodetectors (see, e.g., copending U.S. application

-102-

Serial No. 08/990,103), for detection of tumors (see, e.g., U.S. application Serial No. 08/908,909, or in bioluminescent novelty items (see, U.S. application Serial Nos. 08/597,274 and 08/757,046.

- These compositions can be used in a variety of methods and systems,
- 5 such as included in conjunction with diagnostic systems for the *in vivo* detection of neoplastic tissues and other tissues, such as those methods described in detail below. These methods and products include any known to those of skill in the art in which luciferase is used, including, but not limited to U.S. application Serial Nos. 08/757,046, 08/597,274 and 08/990,103, U.S.
- 10 Patent No. 5,625,048; International patent application Publication Nos. WO 95/21191; WO 96/23810; WO 96/27675; WO 97/26333; WO 97/28261; WO 97/41228; and WO 98/02571).

## 2. *Renilla* luciferase compositions

- The DNA encoding the *Renilla mulleri* luciferase is used to produce the
- 15 encoded luciferase, which has diagnostic applications as well as use as a component of the bioluminescence generating systems as described herein, such as in beverages, and methods of diagnosis of neoplasia and in the diagnostic chips described herein. These methods and products include any known to those of skill in the art in which luciferase is used, including, but not
- 20 limited to, U.S. application Serial No. 08/757,046, 08/597,274 and 08/990,103, U.S. Patent No. 5,625,048; International patent application Publication Nos. WO 95/21191; WO 96/23810; WO 96/27675; WO 97/26333; WO 97/28261; WO 97/41228; and WO 98/02571).

- In other embodiments, the *Renilla mulleri* luciferase and the remaining
- 25 components may be packaged as separate compositions, that, upon mixing, glow. For example, a composition containing *Renilla mulleri* luciferase may be provided separately from, and for use with, an a separate composition containing a bioluminescence substrate and bioluminescence activator. In another instance, luciferase and luciferin compositions may be separately
- 30 provided and the bioluminescence activator may be added after, or simultaneously with, mixing of the other two compositions.

-103-

### 3. *Renilla* GFP compositions

Compositions containing a *Renilla* GFP or GFP peptide are provided. The compositions can take any of a number of forms, depending on the intended method of use therefor. In certain embodiments, for example, the compositions  
5 contain a *Renilla* GFP or GFP peptide, preferably *Renilla mulleri* GFP or *Renilla reniformis* GFP peptide, formulated for use in luminescent novelty items, immunoassays, FRET and FET assays. The compositions may also be used in conjunction with multi-well assay devices containing integrated photodetectors, such as those described herein.

10 Compositions that contain a *Renilla mulleri* GFP or GFP peptide and at least one component of a bioluminescence-generating system, preferably a luciferase, luciferin or a luciferase and a luciferin, are provided. In preferred embodiments, the luciferase/luciferin bioluminescence- generating system is selected from those isolated from: an insect system, a coelenterate system, a  
15 ctenophore system, a bacterial system, a mollusk system, a crustacea system, a fish system, an annelid system, and an earthworm system. Presently preferred bioluminescence- generating systems are those isolated from *Renilla*, *Aequorea*, and *Vargula*.

In more preferred embodiments, the bioluminescence-generating system  
20 component is a *Renilla mulleri* luciferase having the amino acid sequence set forth in SEQ ID No. 18. These compositions can be used in a variety of methods and systems, such as included in conjunction with diagnostic systems for the *in vivo* detection of neoplastic tissues and other tissues, such as those methods described in detail below.

25 These methods and products include any known to those of skill in the art in which luciferase is used, including, but not limited to U.S. application Serial No. 08/757,046, 08/597,274 and 08/990,103, U.S. Patent No. 5,625,048; International patent application Publication Nos. WO 95/21191; WO 96/23810; WO 96/27675; WO 97/26333; WO 97/28261; WO 97/41228; and  
30 WO 98/02571).

-104-

#### 4. Conjugates

The conjugates that are provided herein contain a targeting agent, such as a tissue specific or tumor specific monoclonal antibody or fragment thereof linked either directly or via a linker to a targeted agent, a *Renilla* GFP, *Renilla* *mulleri* or *Gaussia* luciferase and other luciferases (including photoproteins or luciferase enzymes) or a luciferin. The targeted agent may be coupled to a microcarrier. The linking is effected either chemically, by recombinant expression of a fusion protein in instances when the targeted agent is a protein, and by combinations of chemical and recombinant expression. The targeting agent is one that will preferentially bind to a selected tissue or cell type, such as a tumor cell surface antigen or other tissue specific antigen.

Methods for preparing conjugates are known to those of skill in the art. For example, aequorin that is designed for conjugation and conjugates containing such aequorin have been produced [see, e.g., International PCT application No. WO 94/18342; see, also Smith et al. (1995) in American Biotechnology Laboratory]. Aequorin has been conjugated to an antibody molecule by means of a sulfhydryl-reacting binding agent (Stultz et al. (1992) Use of Recombinant Biotinylated Apoequorin from *Escherichia coli*. Biochemistry 31, 1433-1442). Such methods may be adapted for use herein to produce the luciferase coupled to protein or other such molecules, which are useful as targeting agents. *Vargula* luciferase has also been linked to other molecules [see, e.g., Japanese application No. JP 5064583, March 19, 1993]. Such methods may be adapted for use herein to produce luciferase coupled to molecules that are useful as targeting agents.

The conjugates can be employed to detect the presence of or quantitate a particular antigen in a biological sample by direct correlation to the light emitted from the bioluminescent reaction.

As an alternative, a component of the bioluminescence generating system may be modified for linkage, such as by addition of amino acid residues that are particularly suitable for linkage to the selected substrate. This can be readily effected by modifying the DNA and expressing such modified DNA to produce luciferase with additional residues at the N- or C-terminus.

-105-

Methods for preparing conjugates are known to those of skill in the art. For example, aequorin that is designed for conjugation and conjugates containing such aequorin have been produced [see, e.g., International PCT application No. WO 94/18342; see, also Smith et al. (1995) in American Biotechnology Laboratory]. Aequorin has been conjugated to an antibody molecule by means of a sulfhydryl-reacting binding agent (Stultz et al. (1992) Use of Recombinant Biotinylated Apoequorin from *Escherichia coli*. Biochemistry 31, 1433-1442). Such methods may be adapted for use herein to produce aequorin coupled to protein or other such molecules, which are useful as targeting agents.

10 *Vargula* luciferase has also been linked to other molecules [see, e.g., Japanese application No. JP 5064583, March 19, 1993]. Such methods may be adapted for use herein to produce aequorin coupled to protein or other such molecules, which are useful as targeting agents.

Aequorin-antibody conjugates have been employed to detect the presence of or quantitate a particular antigen in a biological sample by direct correlation to the light emitted from the bioluminescent reaction.

15

Selection of the system depends upon factors such as the desired color and duration of the bioluminescence desired as well as the particular item. Selection of the targeting agent primarily depends upon the type and characteristics of neoplasia or tissue to be visualized and the setting in which visualization will be performed. For example, the luciferase isolated from *Aristostomias* emits red light, which is particularly beneficial for preoperative diagnosis because the red light is detectable through tissue using a photomultiplier.

20

#### 25 a. Linkers

Any linker known to those of skill in the art may be used herein. Other linkers are suitable for incorporation into chemically produced conjugates. Linkers that are suitable for chemically linked conjugates include disulfide bonds, thioether bonds, hindered disulfide bonds, and covalent bonds between free reactive groups, such as amine and thiol groups. These bonds are produced using heterobifunctional reagents to produce reactive thiol groups on one or both of the polypeptides and then reacting the thiol groups on one

30

-106-

polypeptide with reactive thiol groups or amine groups to which reactive maleimido groups or thiol groups can be attached on the other. Other linkers include, acid cleavable linkers, such as bismaleimideoxy propane, acid labile-transferrin conjugates and adipic acid dihydrazide, that would be cleaved in  
5 more acidic intracellular compartments; cross linkers that are cleaved upon exposure to UV or visible light and linkers, such as the various domains, such as C<sub>H</sub>1, C<sub>H</sub>2, and C<sub>H</sub>3, from the constant region of human IgG<sub>1</sub> (see, Batra *et al.* (1993) Molecular Immunol. 30:379-386). In some embodiments, several linkers may be included in order to take advantage of desired properties of each linker.

10

Chemical linkers and peptide linkers may be inserted by covalently coupling the linker to the TA and the targeted agent. The heterobifunctional agents, described below, may be used to effect such covalent coupling. Peptide linkers may also be linked by expressing DNA encoding the linker and  
15 TA, linker and targeted agent, or linker, targeted agent and TA as a fusion protein.

Flexible linkers and linkers that increase solubility of the conjugates are contemplated for use, either alone or with other linkers are contemplated herein.

Numerous heterobifunctional cross-linking reagents that are used to form  
20 covalent bonds between amino groups and thiol groups and to introduce thiol groups into proteins, are known to those of skill in this art (see, *e.g.*, the PIERCE CATALOG, ImmunoTechnology Catalog & Handbook, 1992-1993, which describes the preparation of and use of such reagents and provides a commercial source for such reagents; see, also, *e.g.*, Cumber *et al.* (1992) Bioconjugate Chem. 3:397-401; Thorpe *et al.* (1987) Cancer Res. 47:5924-5931; Gordon *et al.* (1987) Proc. Natl. Acad. Sci. 84:308-312; Walden *et al.* (1986) J. Mol. Cell Immunol. 2:191-197; Carlsson *et al.* (1978) Biochem. J. 173: 723-737; Mahan *et al.* (1987) Anal. Biochem. 162:163-170; Wawryznaczk *et al.* (1992) Br. J. Cancer 66:361-366; Fattom *et al.* (1992)  
25 Infection & Immun. 60:584-589). These reagents may be used to form covalent bonds between the TA and targeted agent. These reagents include, but are not limited to: N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP;

30



-107-

- disulfide linker); sulfosuccinimidyl 6-[3-(2-pyridyldithio)propionamido]hexanoate (sulfo-LC-SPDP); succinimidylloxycarbonyl- $\alpha$ -methyl benzyl thiosulfate (SMBT, hindered disulfate linker); succinimidyl 6-[3-(2-pyridyldithio) propionamido]-hexanoate (LC-SPDP); sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC); succinimidyl 3-(2-pyridyldithio)butyrate (SPDB; hindered disulfide bond linker); sulfosuccinimidyl 2-(7-azido-4-methylcoumarin-3-acetamide) ethyl-1,3'-dithiopropionate (SAED); sulfo-succinimidyl 7-azido-4-methylcoumarin-3-acetate (SAMCA); sulfosuccinimidyl 6-[ $\alpha$ -methyl- $\alpha$ -(2-pyridyldithio)toluamido]hexanoate (sulfo-LC-SMPT); 1,4-di-[3'-(2'-pyridyldithio)propionamido]butane (DPDPB); 4-succinimidylloxycarbonyl- $\alpha$ -methyl- $\alpha$ -(2-pyridylthio)toluene (SMPT, hindered disulfate linker); sulfosuccinimidyl 6-[ $\alpha$ -methyl- $\alpha$ -(2-pyridyldithio)toluamido]hexanoate (sulfo-LC-SMPT); *m*-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS); *m*-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (sulfo-MBS); N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB; thioether linker); sulfosuccinimidyl(4-iodoacetyl)amino benzoate (sulfo-SIAB); succinimidyl(4-*p*-maleimidophenyl)butyrate (SMPB); sulfosuccinimidyl(4-*p*-maleimidophenyl)butyrate (sulfo-SMPB); azidobenzoyl hydrazide (ABH).

- Acid cleavable linkers, photocleavable and heat sensitive linkers may also be used, particularly where it may be necessary to cleave the targeted agent to permit it to be more readily accessible to reaction.

- Acid cleavable linkers include, but are not limited to, bismaleimideoxy propane; and adipic acid dihydrazide linkers (see, e.g., Fattom et al. (1992) Infection & Immun. 60:584-589) and acid labile transferrin conjugates that contain a sufficient portion of transferrin to permit entry into the intracellular transferrin cycling pathway (see, e.g., Welhöner et al. (1991) J. Biol. Chem. 266:4309-4314).

- Photocleavable linkers are linkers that are cleaved upon exposure to light (see, e.g., Goldmacher et al. (1992) Bioconj. Chem. 3:104-107, which linkers are herein incorporated by reference), thereby releasing the targeted agent upon exposure to light. Photocleavable linkers that are cleaved upon exposure to light are known (see, e.g., Hazum et al. (1981) in Pept., Proc. Eur.

-108-

Pept. Symp., 16th, Brunfeldt, K (Ed), pp. 105-110, which describes the use of a nitrobenzyl group as a photocleavable protective group for cysteine; Yen et al. (1989) Makromol. Chem 190:69-82, which describes water soluble photocleavable copolymers, including hydroxypropylmethacrylamide copolymer, glycine copolymer, fluorescein copolymer and methylrhodamine copolymer; Goldmacher et al. (1992) Bioconj. Chem. 3:104-107, which describes a cross-linker and reagent that undergoes photolytic degradation upon exposure to near UV light (350 nm); and Senter et al. (1985) Photochem. Photobiol 42:231-237, which describes nitrobenzyloxycarbonyl chloride cross linking reagents that produce photocleavable linkages), thereby releasing the targeted agent upon exposure to light. Such linkers would have particular use in treating dermatological or ophthalmic conditions that can be exposed to light using fiber optics. After administration of the conjugate, the eye or skin or other body part can be exposed to light, resulting in release of the targeted moiety from the conjugate. Such photocleavable linkers are useful in connection with diagnostic protocols in which it is desirable to remove the targeting agent to permit rapid clearance from the body of the animal.

#### b. Targeting Agents

Targeting agents include any agent that will interact with and localize the targeted agent cells in a tumor or specialized tissue [targeted tissue]. Such agents include any agent that specifically interacts with a cell surface protein or receptor that is present at sufficiently higher concentrations or amounts on the targeted tissue, whereby, when contacted with an appropriate bioluminescence generating reagent and activators produces light. These agents include, but are not limited to, growth factors, preferentially modified to not internalize, methotrexate, and antibodies, particularly, antibodies raised against tumor specific antigens. A plethora of tumor-specific antigens have been identified from a number of human neoplasms.

#### Anti-tumor Antigen Antibodies

Polyclonal and monoclonal antibodies may be produced against selected antigens. Alternatively, many such antibodies are presently available. An exemplary list of antibodies and the tumor antigen for which each has been

-109-

directed against is provided in U.S. application Serial No., which is incorporated by reference in its entirety. It is contemplated that any of the antibodies listed may be conjugated with a bioluminescence generating component following the methods provided herein.

- 5           Among the preferred antibodies for use in the methods herein are those of human origin or, more preferably, are humanized monoclonal antibodies. These are preferred for diagnosis of humans.

#### Preparation of the conjugates

- Any method for linking proteins may be used. For example, methods for  
10   linking a luciferase to an antibody is described in U.S. Patent No. 5,486,455. As noted above, the targeting agent and luciferin or luciferase may be linked directly, such as through covalent bonds, i.e., sulfhydryl bonds or other suitable bonds, or they may be linked through a linker. There may be more than one luciferase or luciferin per targeting agent, or more than one targeting agent per  
15   luciferase or luciferin.

- Alternatively, an antibody, or F(Ab)<sub>2</sub> antigen-binding fragment thereof or other protein targeting agent may be fused (directly or via a linking peptide) to the luciferase using recombinant DNA technology. For example, the DNA encoding any of the anti-tumor antibodies of Table 3 may be ligated in the same  
20   translational reading frame to DNA encoding any of the above-described luciferases; e.g., SEQ ID NOs. 1-14 and inserted into an expression vector. The DNA encoding the recombinant antibody-luciferase fusion may be introduced into an appropriate host, such as bacteria or yeast, for expression.

#### 5. Formulation of the compositions for use in the diagnostic systems

- 25           In most embodiments, the *Renilla* GFPS and components of the diagnostic systems provided herein, such as *Renilla mulleri* luciferase, are formulated into two compositions: a first composition containing the conjugate; and a second composition containing the remaining components of the bioluminescence generating system. The compositions are formulated in any  
30   manner suitable for administration to an animal, particularly a mammal, and more particularly a human. Such formulations include those suitable for topical,

-110-

local, enteric, parenteral, intracystal, intracutaneous, intravitreal, subcutaneous, intramuscular, or intravenous administration.

For example, the conjugates, which in preferred embodiments, are a  
5 targeting agent linked to a luciferase (or photoprotein) are formulated for systemic or local administration. The remaining components are formulated in a separate second composition for topical or local application. The second composition will typically contain any other agents, such as spectral shifters that will be included in the reaction. It is preferred that the components of the  
10 second composition are formulated in a time release manner or in some other manner that prevents degradation and/or interaction with blood components.

a. The first composition: formulation of the conjugates

As noted above, the conjugates either contain a luciferase or luciferin and a targeting agents. The preferred conjugates are formed between a  
15 targeting agent and a luciferase, particularly the *Gaussia*, *Renilla mulleri* or *Pleuromamma* luciferase. The conjugates may be formulated into pharmaceutical compositions suitable for topical, local, intravenous and systemic application. Effective concentrations of one or more of the conjugates are mixed with a suitable pharmaceutical carrier or vehicle. The concentrations  
20 or amounts of the conjugates that are effective requires delivery of an amount, upon administration, that results in a sufficient amount of targeted moiety linked to the targeted cells or tissue whereby the cells or tissue can be visualized during the surgical procedure. Typically, the compositions are formulated for single dosage administration. Effective concentrations and amounts may be  
25 determined empirically by testing the conjugates in known in vitro and in vivo systems, such as those described here; dosages for humans or other animals may then be extrapolated therefrom.

Upon mixing or addition of the conjugate(s) with the vehicle, the resulting mixture may be a solution, suspension, emulsion or the like. The form  
30 of the resulting mixture depends upon a number of factors, including the intended mode of administration and the solubility of the conjugate in the selected carrier or vehicle. The effective concentration is sufficient for targeting

-111-

a sufficient amount of targeted agent to the site of interest, whereby when combined with the remaining reagents during a surgical procedure the site will glow. Such concentration or amount may be determined based upon in vitro and/or in vivo data, such as the data from the mouse xenograft model for

- 5 tumors or rabbit ophthalmic model. If necessary, pharmaceutically acceptable salts or other derivatives of the conjugates may be prepared.

Pharmaceutical carriers or vehicles suitable for administration of the conjugates provided herein include any such carriers known to those skilled in the art to be suitable for the particular mode of administration.

- 10 In addition, the conjugates may be formulated as the sole pharmaceutically ingredient in the composition or may be combined with other active ingredients.

The conjugates can be administered by any appropriate route, for example, orally, parenterally, intravenously, intradermally, subcutaneously, or topically, in liquid, semi-liquid or solid form and are formulated in a manner

- 15 suitable for each route of administration. Intravenous or local administration is presently preferred. Tumors and vascular proliferative disorders, will typically be visualized by systemic, intradermal or intramuscular, modes of administration.

The conjugate is included in the pharmaceutically acceptable carrier in an amount sufficient to produce detectable tissue and to not result in undesirable side effects on the patient or animal. It is understood that number and degree of side effects depends upon the condition for which the conjugates are administered. For example, certain toxic and undesirable side effects are tolerated when trying to diagnose life-threatening illnesses, such as tumors, that

- 25 would not be tolerated when diagnosing disorders of lesser consequence.

The concentration of conjugate in the composition will depend on absorption, inactivation and excretion rates thereof, the dosage schedule, and amount administered as well as other factors known to those of skill in the art. Typically an effective dosage should produce a serum concentration of active ingredient of from about 0.1 ng/ml to about 50-1000  $\mu$ g/ml, preferably 50-100  $\mu$ g/ml. The pharmaceutical compositions typically should provide a dosage of from about 0.01 mg to about 100 - 2000 mg of conjugate, depending upon the

- 30

-112-

conjugate selected, per kilogram of body weight per day. Typically, for intravenous administration a dosage of about between 0.05 and 1 mg/kg should be sufficient. Local application for, such as visualization of ophthalmic tissues or local injection into joints, should provide about 1 ng up to 1000  $\mu$ g, preferably about 1  $\mu$ g to about 100  $\mu$ g, per single dosage administration. It is understood that the amount to administer will be a function of the conjugate selected, the indication, and possibly the side effects that will be tolerated. Dosages can be empirically determined using recognized models.

The active ingredient may be administered at once, or may be divided into a number of smaller doses to be administered at intervals of time. It is understood that the precise dosage and duration of administration is a function of the disease condition being diagnosed and may be determined empirically using known testing protocols or by extrapolation from in vivo or in vitro test data. It is to be noted that concentrations and dosage values may also vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed compositions.

Solutions or suspensions used for parenteral, intradermal, subcutaneous, or topical application can include any of the following components: a sterile diluent, such as water for injection, saline solution, fixed oil, polyethylene glycol, glycerine, propylene glycol or other synthetic solvent; antimicrobial agents, such as benzyl alcohol and methyl parabens; antioxidants, such as ascorbic acid and sodium bisulfite; chelating agents, such as ethylenediaminetetraacetic acid (EDTA); buffers, such as acetates, citrates and phosphates; and agents for the adjustment of tonicity such as sodium chloride or dextrose. Parental preparations can be enclosed in ampules, disposable syringes or multiple dose vials made of glass, plastic or other suitable material.

If administered intravenously, suitable carriers include physiological saline or phosphate buffered saline (PBS), and solutions containing thickening and

-113-

solubilizing agents, such as glucose, polyethylene glycol, and polypropylene glycol and mixtures thereof. Liposomal suspensions may also be suitable as pharmaceutically acceptable carriers. These may be prepared according to methods known to those skilled in the art.

5           The conjugates may be prepared with carriers that protect them against rapid elimination from the body, such as time release formulations or coatings. Such carriers include controlled release formulations, such as, but not limited to, implants and microencapsulated delivery systems, and biodegradable, biocompatible polymers, such as ethylene vinyl acetate, polyanhydrides,  
10 polyglycolic acid, polyorthoesters, polyacetic acid and others.

          The conjugates may be formulated for local or topical application, such as for topical application to the skin and mucous membranes, such as in the eye, in the form of gels, creams, and lotions and for application to the eye or for intracisternal or intraspinal application. Such solutions, particularly those  
15 intended for ophthalmic use, may be formulated as 0.01% -10% isotonic solutions, pH about 5-7, with appropriate salts. The ophthalmic compositions, may also include additional components, such as hyaluronic acid. The conjugates may be formulated as aerosols for topical application (see, e.g., U.S. Patent Nos. 4,044,126, 4,414,209, and 4,364,923).

20           Also, the compositions for activation of the conjugate in vivo during surgical procedures may be formulated as an aerosol. These compositions contain the activators and also the remaining bioluminescence generating agent, such as luciferin, where the conjugate targets a luciferase, or a luciferase, where the conjugate targets a luciferin, such as coelenterazine.

25           If oral administration is desired, the conjugate should be provided in a composition that protects it from the acidic environment of the stomach. For example, the composition can be formulated in an enteric coating that maintains its integrity in the stomach and releases the active compound in the intestine. Oral compositions will generally include an inert diluent or an edible carrier and  
30 may be compressed into tablets or enclosed in gelatin capsules. For the purpose of oral administration, the active compound or compounds can be incorporated with excipients and used in the form of tablets, capsules or

-114-

troches. Pharmaceutically compatible binding agents and adjuvant materials can be included as part of the composition.

Tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder, such as microcrystalline cellulose, gum tragacanth and gelatin; an excipient such as starch and lactose, a disintegrating agent such as, but not limited to, alginic acid and corn starch; a lubricant such as, but not limited to, magnesium stearate; a glidant, such as, but not limited to, colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; and a flavoring agent such as peppermint, methyl salicylate, and fruit flavoring.

When the dosage unit form is a capsule, it can contain, in addition to material of the above type, a liquid carrier such as a fatty oil. In addition, dosage unit forms can contain various other materials which modify the physical form of the dosage unit, for example, coatings of sugar and other enteric agents. The conjugates can also be administered as a component of an elixir, suspension, syrup, wafer, chewing gum or the like. A syrup may contain, in addition to the active compounds, sucrose as a sweetening agent and certain preservatives, dyes and colorings and flavors.

The active materials can also be mixed with other active materials that do not impair the desired action, or with materials that supplement the desired action, such as cis-platin for treatment of tumors.

Finally, the compounds may be packaged as articles of manufacture containing packaging material, one or more conjugates or compositions as provided herein within the packaging material, and a label that indicates the indication for which the conjugate is provided.

**b. The second composition**

The second composition will include the remaining components of the bioluminescence generating reaction. In preferred embodiments in which these components are administered systemically, the remaining components include the luciferin or substrate, and optionally additional agents, such as spectral shifters, particularly the GFPs provided herein. These components, such as the luciferin, can be formulated as described above for the conjugates. In some



-115-

embodiments, the luciferin or luciferase in this composition will be linked to a protein carrier or other carrier to prevent degradation or dissolution into blood cells or other cellular components.

For embodiments, in which the second composition is applied locally or  
5 topically, they can be formulated in a spray or aerosol or other suitable means for local or topical application.

In certain embodiments described herein, all components, except an activator are formulated together, such as by encapsulation in a time release formulation that is targeted to the tissue. Upon release the composition will  
10 have been localized to the desired site, and will begin to glow.

In practice, the two compositions can be administered simultaneously or sequentially. Typically, the first composition, which contains the conjugate is administered first, generally an hour or two before the surgery, and the second composition is then administered, either pre-operatively or during surgery.

15 The conjugates that are provided herein contain a targeting agent, such as a tissue specific or tumor specific monoclonal antibody or fragment thereof linked either directly or via a linker to a targeted agent, a luciferase (including photoproteins or luciferase enzymes) or a luciferin. The targeted agent may be coupled to a microcarrier. The linking is effected either chemically, by  
20 recombinant expression of a fusion protein in instances when the targeted agent is a protein, and by combinations of chemical and recombinant expression. The targeting agent is one that will preferentially bind to a selected tissue or cell type, such as a tumor cell surface antigen or other tissue specific antigen.

Methods for preparing conjugates are known to those of skill in the art.  
25 For example, aequorin that is designed for conjugation and conjugates containing such aequorin have been produced [see, e.g., International PCT application No. WO 94/18342; see, also Smith et al. (1995) in American Biotechnology Laboratory]. Aequorin has been conjugated to an antibody molecule by means of a sulfhydryl-reacting binding agent (Stultz et al. (1992)  
30 Use of Recombinant Biotinylated Apoaequorin from *Escherichia coli*. Biochemistry 31, 1433-1442). Such methods may be adapted for use herein to produce aequorin coupled to protein or other such molecules, which are useful

-116-

as targeting agents. *Vargula* luciferase has also been linked to other molecules [see, e.g., Japanese application No. JP 5064583, March 19, 1993]. Such methods may be adapted for use herein to produce aequorin coupled to protein or other such molecules, which are useful as targeting agents.

- 5        Aequorin-antibody conjugates have been employed to detect the presence of or quantitate a particular antigen in a biological sample by direct correlation to the light emitted from the bioluminescent reaction.

- 10        As an alternative, the *Renilla* GFP or *Renilla mulleri* or *Gaussia* luciferase or a component of the bioluminescence generating system may be modified for linkage, such as by addition of amino acid residues that are particularly suitable for linkage to the selected substrate. This can be readily effected by modifying the DNA and expressing such modified DNA to produce luciferase with additional residues at the N- or C-terminus.

- 15        Selection of the system depends upon factors such as the desired color and duration of the bioluminescence desired as well as the particular item. Selection of the targeting agent primarily depends upon the type and characteristics of neoplasia or tissue to be visualized and the setting in which visualization will be performed.

- 20        c.        Practice of the reactions in combination with targeting agents

- 25        The particular manner in which each bioluminescence system will be combined with a selected targeting agent will be a function of the agent and the neoplasia or tissue to be visualized. In general, however, a luciferin, *Renilla* GFP, *Renilla mulleri*, *Pleuromamma* or *Gaussia* luciferase or other luciferase, of the reaction will be conjugated to the targeting agent, administered to an animal prior to surgery. During the surgery, the tissues of interest are contacted with the remaining component(s) of a bioluminescence generating system. Any tissue to which or with which the targeting agent reacts will glow.

-117-

Any color of visible light produced by a bioluminescence generating system is contemplated for use in the methods herein. Preferably the visible light is a combination of blue, green and/or red light of varying intensities and wavelengths. For visualizing neoplasia or specialty tissues through mammalian  
5 tissues or tumors deeply embedded in tissue, longer wavelengths of visible light, i.e., red and near infrared light, is preferred because wavelengths of near infrared light of about 700-1300 nm are known to penetrate soft tissue and bone [e.g., see U.S. Patent No. 4,281,645].

In other embodiments, the conjugate can be applied to the tissues during  
10 surgery, such as by spraying a sterile solution over the tissues, followed by application of the remaining components. Tissues that express the targeted antigen will glow.

The reagents may be provided in compositions, such as suspensions, as powders, as pastes or any in other suitable sterile form. They may be provided  
15 as sprays, aerosols, or in any suitable form. The reagents may be linked to a matrix, particularly microbeads suitable for in vivo use and of size that they pass through capillaries. Typically all but one or more, though preferably all but one, of the components necessary for the reaction will be mixed and provided together; reaction will be triggered contacting the mixed component(s) with the  
20 remaining component(s), such as by adding  $\text{Ca}^{2+}$ , FMN with reductase,  $\text{FMNH}_2$ , ATP, air or oxygen.

In preferred embodiments the luciferase or luciferase/luciferin will be provided in combination with the targeting agent before administration to the patient. The targeting agent conjugate will then be contacted in vivo with the  
25 remaining components. As will become apparent herein, there are a multitude of ways in which each system may be combined with a selected targeting agent.

#### J. COMBINATIONS

In addition, the above-described *Pleuromamma*, *Gaussia* or *Renilla*  
30 luciferases and/or *Renilla* and *Ptilosarcus* GFPs can be used in combination with articles of manufacture to produce novelty items. Such items and methods for preparation are described in detail in copending U.S. application Serial Nos.

-118-

08/597,274 and 08/757,046. The luciferases and/or GFPs provided herein may be used in the methods and items as provided in the copending applications.

These novelty items, which are articles of manufacture, are designed for entertainment, recreation and amusement, and include, but are not limited to:

- 5 toys, particularly squirt guns, toy cigarettes, toy "Halloween" eggs, footbags and board/card games; finger paints and other paints, slimy play material; textiles, particularly clothing, such as shirts, hats and sports gear suits, threads and yarns; bubbles in bubble making toys and other toys that produce bubbles; balloons; figurines; personal items, such as bath powders, body lotions, gels,
- 10 powders and creams, nail polishes, cosmetics including make-up, toothpastes and other dentifrices, soaps, body paints, and bubble bath; items such as inks, paper; foods, such as gelatins, icings and frostings; fish food containing luciferins and transgenic fish, particularly transgenic fish that express a luciferase; plant food containing a luciferin or luciferase, preferably a luciferin for
- 15 use with transgenic plants that express luciferase; and beverages, such as beer, wine, champagne, soft drinks, and ice cubes and ice in other configurations; fountains, including liquid "fireworks" and other such jets or sprays or aerosols of compositions that are solutions, mixtures, suspensions, powders, pastes, particles or other suitable form.

- 20 Any article of manufacture that can be combined with a bioluminescence-generating system as provided herein and thereby provide entertainment, recreation and/or amusement, including use of the items for recreation or to attract attention, such as for advertising goods and/or services that are associated with a logo or trademark is contemplated herein. Such uses
- 25 may be in addition to or in conjunction with or in place of the ordinary or normal use of such items. As a result of the combination, the items glow or produce, such as in the case of squirt guns and fountains, a glowing fluid or spray of liquid or particles. K.

#### METHODS OF USE

##### 1. Methods for diagnosis of neoplasms and other tissues

- 30 Methods for diagnosis and visualization of tissues *in vivo* or *in situ*, preferably neoplastic tissue, using compositions containing a *Renilla mulleri* or *Ptilosarcus* GFP and/or a *Renilla mulleri*, *Pleuromamma* or *Gaussia* luciferase are

-119-

provided. For example, the *Renilla mulleri* GFP protein can be used in conjunction with diagnostic systems that rely on bioluminescence for visualizing tissues *in situ*, such as those described in co-pending application Serial No. 08/908,909. The systems are particularly useful for visualizing and detecting

5 neoplastic tissue and specialty tissue, such as during non-invasive and invasive procedures. The systems include compositions containing conjugates that include a tissue specific, particularly a tumor-specific, targeting agent linked to a targeted agent, such as a *Renilla mulleri* GFP, a luciferase or luciferin. The systems also include a second composition that contains the remaining

10 components of a bioluminescence generating reaction and/or the GFP. In some embodiments, all components, except for activators, which are provided *in situ* or are present in the body or tissue, are included in a single composition.

In particular, the diagnostic systems include two compositions. A first composition that contains conjugates that, in preferred embodiments, include

15 antibodies directed against tumor antigens conjugated to a component of the bioluminescence generating reaction, a luciferase or luciferin, preferably a luciferase are provided. In certain embodiments, conjugates containing tumor-specific targeting agents are linked to luciferases or luciferins. In other

embodiments, tumor-specific targeting agents are linked to microcarriers that

20 are coupled with, preferably more than one of the bioluminescence generating components, preferably more than one luciferase molecule.

The second composition contains the remaining components of a bioluminescence generating system, typically the luciferin or luciferase substrate. In some embodiments, these components, particularly the luciferin

25 are linked to a protein, such as a serum albumin, or other protein carrier. The carrier and time release formulations, permit systemically administered components to travel to the targeted tissue without interaction with blood cell components, such as hemoglobin that deactivates the luciferin or luciferase.

-120-

## 2. Methods of diagnosing diseases

Methods for diagnosing diseases, particularly infectious diseases, using chip methodology, a luciferase/luciferin bioluminescence-generating system, including a *Gaussia*, *Pleuromamma* or *Renilla mulleri* luciferase and/or a

5 *Ptilosarcus* or *Renilla mulleri* GFP, are provided. In particular, the chip includes an integrated photodetector that detects the photons emitted by the bioluminescence-generating system and/or GFP.

In one embodiment, the chip is made using an integrated circuit with an array, such as an X-Y array, of photodetectors, such as that described in co-  
10 pending U.S. application Serial No. 08/990,103. The surface of circuit is treated to render it inert to conditions of the diagnostic assays for which the chip is intended, and is adapted, such as by derivatization for linking molecules, such as antibodies. A selected antibody or panel of antibodies, such as an antibody specific for particularly bacterial antigen, is affixed to the surface of  
15 the chip above each photodetector. After contacting the chip with a test sample, the chip is contacted with a second antibody linked to the GFP, such as the *Renilla* GFP, to form a chimeric antibody- GFP fusion protein or an antibody linked to a component of a bioluminescence generating system, such as a *Pleuromamma*, *Gaussia* or *R. mulleri* luciferase. The antibody is specific for the  
20 antigen. The remaining components of the bioluminescence generating reaction are added, and, if any of the antibodies linked to a component of a bioluminescence generating system are present on the chip, light will be generated and detected by the adjacent photodetector. The photodetector is operatively linked to a computer, which is programmed with information  
25 identifying the linked antibodies, records the event, and thereby identifies antigens present in the test sample.

## 3. Methods for generating chimeric *Renilla* or *Ptilosarcus* GFP, *Renilla mulleri* luciferase, *Pleuromamma* luciferase and *Gaussia* luciferase fusion proteins

30 Methods for generating chimeric GFP and luciferase fusion proteins are provided. The methods include linking DNA encoding a gene of interest, or portion thereof, to DNA encoding a GFP or luciferase provided herein in the

-121-

same translational reading frame. The encoded-protein of interest may be linked in-frame to the amino- or carboxyl-terminus of the GFP or luciferase. The DNA encoding the chimeric protein is then linked in operable association with a promoter element of a suitable expression vector. Alternatively, the promoter element can be obtained directly from the targeted gene of interest and the promoter-containing fragment linked upstream from the GFP or luciferase coding sequence to produce chimeric GFP proteins.

For example, a chimeric fusion containing the *Gaussia* luciferase encoding DNA linked to the N-terminal portion of a cellulose binding domain is provided (see, SEQ ID Nos. 21 and 22).

#### 4. Cell-based assays for identifying compounds

Methods for identifying compounds using recombinant cells that express heterologous DNA encoding a *Renilla mulleri* or *Ptilosarcus* GFP under the control of a promoter element of a gene of interest are provided. The recombinant cells can be used to identify compounds or ligands that modulate the level of transcription from the promoter of interest by measuring GFP-mediated fluorescence. Recombinant cells expressing chimeric GFPs may also be used for monitoring gene expression or protein trafficking, or determining the cellular localization of the target protein by identifying localized regions of GFP-mediated fluorescence within the recombinant cell.

#### L. KITS

Kits may be prepared containing the *Gaussia*, *Pleuromamma* or *Renilla mulleri* luciferase or the *Renilla* and *Ptilosarcus* GFPs for use in diagnostic and immunoassay methods and with the novelty items, including those described herein.

In one embodiment, the kits contain appropriate reagents and an article of manufacture for generating bioluminescence in combination with the article. These kits, for example, can be used with a bubble-blowing or producing toy or with a squirt gun. These kits can also include a reloading or charging cartridge. In another embodiment, the kits are used for detecting and visualizing neoplastic tissue and other tissues and include a first composition that contains the luciferase and/or *Renilla mulleri* or *Ptilosarcus* GFP and at least one

-122-

component of a bioluminescence generating system, and a second that contains the activating composition, which contains the remaining components of the bioluminescence generating system and any necessary activating agents.

In other embodiments, the kits are used for detecting and identifying  
5 diseases, particularly infectious diseases, using multi-well assay devices and include a multi-well assay device containing a plurality of wells, each having an integrated photodetector, to which an antibody or panel of antibodies specific for one or more infectious agents are attached, and composition containing a secondary antibody, such as an antibody specific for the infectious agent that is  
10 linked, for example, to a *Renilla mulleri* GFP protein, a chimeric antibody-*Renilla mulleri* GFP fusion protein, F(Ab)<sub>2</sub> antibody fragment-*Renilla mulleri* GFP fusion protein or to such conjugates containing the, for example, *Gaussia* or *Renilla mulleri*, luciferase. A second composition containing the remaining components of a bioluminescence generating system, such as system that emits a  
15 wavelength of light within the excitation range of the GFP, such as species of *Renilla* or *Aequorea*, for exciting the *Renilla mulleri* luciferase, which produces green light that is detected by the photodetector of the device to indicate the presence of the agent.

In further embodiments, the kits contain the components of the  
20 diagnostic systems. The kits comprise compositions containing the conjugates, preferably *Renilla* or *Ptilosarcus* GFP or *Gaussia*, or *Pleuromamma* or *Renilla mulleri* luciferase and remaining bioluminescence generating system components. The first composition in the kit typically contains the targeting agent conjugated to a GFP or luciferase. The second composition, contains at  
25 least the luciferin (substrate) and/or luciferase. Both compositions are formulated for systemic, local or topical application to a mammal. In alternative embodiments, the first composition contains the luciferin linked to a targeting agent, and the second composition contains the luciferase or the luciferase and a GFP.

30 In general, the packaging is non-reactive with the compositions contained therein and where needed should exclude water and or air to the degree those substances are required for the luminescent reaction to proceed.



-123-

Diagnostic applications may require specific packaging. The bioluminescence generating reagents may be provided in pellets, encapsulated as micro or macro-capsules, linked to matrices, preferably biocompatible, more preferably biodegradable matrices, and included in or on articles of manufacture, or as mixtures in chambers within an article of manufacture or in some other configuration. For example, a composition containing luciferase conjugate will be provided separately from, and for use with, a separate composition containing a bioluminescence substrate and bioluminescence activator.

Similarly, the *Renilla* or *Ptilosarcus* GFP, *Pleuromamma*, *Renilla mulleri* or *Gaussia* luciferase or luciferin may be provided in a composition that is a mixture, suspension, solution, powder, paste or other suitable composition separately from or in combination with the remaining components, but in the absence of an activating component. Upon contacting the conjugate, which has been targeted to a selected tissue, with this composition the reaction commences and the tissue glows. In preferred embodiments, the tissue glows green emitting light near 510 nm. The luciferase, GFP and bioluminescence substrate, for example, are packaged to exclude water and/or air, the bioluminescence activator. Upon administration and release at the targeted site, the reaction with salts or other components at the site, including air in the case of surgical procedures, will activate the components.

**1. Dispensing and Packaging Apparatus for Combination with the GFP and Bioluminescent System Components**

The bioluminescence systems, described in detail herein, include at least three components: a bioluminescence substrate [e.g., a luciferin], a luciferase [e.g., a luciferase or photoprotein], preferably *Gaussia*, *Pleuromamma* or *Renilla mulleri* luciferase, and a bioluminescence activator or activators [e.g., molecular oxygen or  $\text{Ca}^{2+}$ ], and optionally a *Renilla* or *Ptilosarcus* GFP. The dispensing and packaging apparatus are configured to keep at least one of the components separate from the remaining components, until generation of bioluminescence is desired. Detailed descriptions of such apparatus are described in copending, commonly owned U.S. application Serial Nos. 08/757,046 and 08/597,274, which are incorporated by reference herein.

-124-

## 2. Capsules, pellets, liposomes, endosomes, vacuoles, micronized particles

In certain embodiments sequestering of the components of one of the compositions from the environment prior to use or provision of the components in particulate form, such as microparticles, may be necessary. Examples of suitable means for such use include encapsulating bioluminescent generating system components in one or micro- [up to about 100  $\mu\text{m}$  in size] or macroparticles [larger than 100  $\mu\text{m}$ ] of material that permits release of the contents, such as by diffusion or by dissolution of the encapsulating material. Microparticles to which a plurality of conjugates can be linked are among the preferred embodiments. The microparticles are biocompatible and preferably of a size that can pass through capillary walls.

Liposomes and other encapsulating vehicles [see, e.g., U.S. Patent No. 4,525,306, which describes encapsulation of compounds in gelatin; U.S. Patent Nos. 4,021,364, 4,225,581, 4,269,821, 4,322,311, 4,324,683, 4,329,332, 4,525,306, 4,963,368 describe encapsulation of biologically active materials in various polymers] known to those of skill in the art, including those discussed herein and known to those of skill in the art [such as soluble paper, see U.S. Patent No. 3,859,125].

### 20 a. Encapsulating vehicles in general

The components of the bioluminescence generating system, except for the oxygen or water or  $\text{Ca}^{2+}$ , depending upon the selected system can be incorporated into encapsulating material, such as liposomes, that protect the contents from the environment until placed into conditions that cause release of the contents into the environment. Encapsulating material contemplated for use herein includes liposomes and other such materials used for encapsulating chemicals, such as drug delivery vehicles.

### b. Encapsulating vehicles -liposomes

For example, liposomes that dissolve and slowly release the components into the medium, such as the blood, which contains dissolved oxygen or  $\text{Ca}^{2+}$  or even ATP for the luciferase system are contemplated herein. They can be formulated in compositions, such as solutions, suspensions, gels, lotions,

-125-

creams, and ointments, for topical application, such as procedures for diagnosing or visualizing melanomas. Liposomes and other slow release encapsulating compositions are well known and can be adapted for use in for slow release delivery of bioluminescence generating components. Typically the GFP, luciferin and/or luciferase will be encapsulated in the absence of oxygen or  $\text{Ca}^{2+}$  or ATP or other activating component. Upon release into the environment or medium containing this component at a suitable concentration, the reaction will proceed and a glow will be produced. Generally the concentrations of encapsulated components should be relatively high, perhaps 0.1 - 1 mg/ml or more, to ensure high enough local concentrations upon release to be visible.

Liposomes or other sustained release delivery system that are formulated in an ointment or sustained release topical vehicle, for example, would be suitable for use in a body paint, lotion. Those formulated as a suspension would be useful as a spray. Numerous ointments and suitable liposome formulations are known [see, e.g., Liposome Technology, Targeted Drug Delivery and Biological Interaction, vol. III, G. Gregoriadis ed., CRC Press, Inc., 1984; U.S. Patent Nos. 5,470,881; 5,366,881; 5,296,231; 5,272,079; 5,225,212; 5,190,762; 5,188,837; 5,188,837; 4,921,757; 4,522,811]. For example, an appropriate ointment vehicle would contain petrolatum, mineral oil and/or anhydrous liquid lanolin. Sustained release vehicles such as liposomes, membrane or contact lens delivery systems, or gel-forming plastic polymers would also be suitable delivery vehicles. Liposomes for topical delivery are well known [see, e.g., U.S. Patent No. 5,296,231; Mezei et al. (1980) "Liposomes -A selective drug delivery system for the topical route of administration, I. lotion dosage form" Life Sciences 26:1473-1477; Mezei et al. (1981) "Liposomes -A selective drug delivery system for the topical route of administration: gel dosage form" Journal of Pharmacy and Pharmacology 34:473-474; Gesztes et al. (1988) "Topical anaesthesia of the skin by liposome -encapsulated tetracaine" Anesthesia and Analgesia 67:1079-1081; Patel (1985) "Liposomes as a controlled-release system", Biochemical Soc. Trans. 13:513-516; Wohlrab et al. (1987) "Penetration kinetics of liposomal hydrocortisone in human skin", Dermatologica 174:18-22].

-126-

Liposomes are microcapsules [diameters typically on the order of less than 0.1 to 20  $\mu\text{m}$ ] that contain selected mixtures and can slowly release their contents in a sustained release fashion. Targeted liposomes or other capsule, particularly a time release coating, that dissolve upon exposure to oxygen, air, moisture, visible or ultraviolet [UV] light or a particular pH or temperature [see, e.g., U.S. Patent No. 4,882,165; Kusumi et al. (1989) Chem. Lett. no.3 433-436; Koch Troels et al. (1990) Bioconjugate Chem. 4:296-304; U.S. Patent No. 5,482,719; U.S. Patent No. 5,411,730; U.S. Patent No. 4,891,043; Straubinger et al. (1983) Cell 32:1069-1079; and Straubinger et al. (1985) FEBS Lttrs. 179:148-154; and Duzgunes et al. in Chapter 11 of the book CELL FUSION, edited by A. E. Sowers; Ellens et al. (1984) Biochemistry 23:1532-1538; Yatvin et al. (1987) Methods in Enzymology 149:77-87] may be used. Liposome formulations for use in baking [see, e.g., U.S. Patent No. 4,999,208] are available. They release their contents when eaten or heated. Such liposomes may be suitable for intravenous or local administration.

Liposomes be prepared by methods known to those of skill in the art [see, e.g., Kimm et al. (1983) Bioch. Bioph. Acta 728:339-398; Assil et al. (1987) Arch Ophthalmol. 105:400; and U.S. Patent No. 4,522,811, and other citations herein and known to those of skill in the art].

Liposomes that are sensitive to low pH [see, e.g., U.S. Patent No. 5,352,448, 5,296,231; 5,283,122; 5,277,913, 4,789,633] are particularly suitable for use with alkaline agents. Upon contact with the low pH detergent or soap composition or a high pH composition, the contents of the liposome will be released. Other components, particularly  $\text{Ca}^+$  or the presence of dissolved  $\text{O}_2$  in the water will cause the components to glow as they are released. Temperature sensitive liposomes are also suitable for use in bath powders for release into the warm bath water.

c. Encapsulating vehicles -gelatin and polymeric vehicles

Macro or microcapsules made of gelatin or other such polymer that dissolve or release their contents on contact with air or light or changes in temperature may also be used to encapsulate components of the bioluminescence generating systems.

-127-

Such microcapsules or macrocapsules may also be conjugated to a targeting agent, e.g., an antibody, such that the GFP or luciferase and bioluminescence generating components are delivered to the target by the antibody and then the components are released to produce a glow.

5       The aequorin system is particularly suitable for this application. It can be encapsulated in suspension or solution or as a paste, or other suitable form, of buffer with sufficient chelating agent, such as EDTA, to prevent discharge of the bioluminescence. Upon exposure of the capsule [microcapsule or microcapsule] to moisture that contains  $\text{Ca}^{2+}$ , such as in a buffer or blood, the  
10       released components will glow.

Thus, encapsulated bioluminescence generating components can be used in combination with a variety of targeting agents and thereby release the luciferase/luciferin, such as the *Renilla mulleri*, *Pleuromamma*, *Ptilosarcus* or *Gaussia* system, which will light upon exposure to air.

15       Other encapsulating containers or vehicles for use with the bioluminescence systems are those that dissolve sufficiently in water to release their contents, or that are readily opened when squeezed in the hand or from which the contents diffuse when mixed with a aqueous mixture. These containers can be made to exclude water, so that the bioluminescent system  
20       components may be desiccated and placed therein. Upon exposure to water, such as in an aqueous composition solution or in the atmosphere, the vehicle dissolves or otherwise releases the contents, and the components react and glow. Similarly, some portion less than all of the bioluminescence generating components may themselves be prepared in pellet form. For example, the  
25       component(s) may be mixed with gelatin or similar hardening agent, poured into a mold, if necessary and dried to a hard, water soluble pellet. The encapsulating containers or vehicles may be formed from gelatin or similar water soluble material that is biocompatible.

d.       Endosomes and vacuoles

30       Vehicles may be produced using endosomes or vacuoles from recombinant host cells in which the *Renilla* or *Ptilosarcus* GFPs or *Renilla mulleri*, *Pleuromamma* or *Gaussia* luciferase is expressed using method known to those

-128-

of skill in the art [see, e.g., U.S. Patent Nos. 5,284,646, 5,342,607, 5,352,432, 5,484,589, 5,192,679, 5,206,161, and 5,360,726]. For example, aequorin that is produced by expression in a host, such as *E. coli*, can be isolated within vesicles, such as endosomes or vacuoles, after protein synthesis. Using routine methods the cells are lysed and the vesicles are released with their contents intact. The vesicles will serve as delivery vehicles. When used they will be charged with a luciferin, such as a coelenterazine, and dissolved oxygen, such as by diffusion, under pressure, or other appropriate means.

10                   e.       **Micronized particles**

The bioluminescence generating system components that are suitable for lyophilization, such as the aequorin photoprotein, the *Renilla* system, *Ptilosarcus*, *Pleuromamma* and the *Gaussia* systems, can be micronized to form fine powder and stored under desiccating conditions, such as with a desiccant.

15   Contact with dissolved oxygen or  $\text{Ca}^{2+}$  in the air or in a mist that can be supplied or in added solution will cause the particles to dissolve and glow.

3.       **Immobilized systems**

a.       **Matrix materials**

In some embodiments, it will be desirable to provide at least the GFPs or one component of the bioluminescence generating system linked to a matrix substrate, which can then be locally or systemically administered. The matrix substrate will be biocompatible. When desired, a mixture or mixtures(s) containing the remaining components, typically a liquid mixture is applied, as by pouring or spraying onto the matrix substrate, to produce a glow. For example, the aequorin photoprotein, including coelenterazine and oxygen, is linked to the substrate. When desired a liquid containing  $\text{Ca}^{2+}$ , such as tap water or, preferably, a liquid mixture containing the  $\text{Ca}^{2+}$  in an appropriate buffer, is contacted, such as by spraying, with the matrix with linked luciferase. Upon contacting in the presence of a GFP the material glows green.

30       In other embodiments, the *Renilla* GFP, *Renilla mulleri* or *Gaussia*, *Pleuromamma* luciferase or other luciferase, such as a *Vargula* luciferase, is linked to the substrate material, and contacted with a liquid mixture containing

-129-

the luciferin in an appropriate buffer. Contacting can be effected by spraying or pouring or other suitable manner. The matrix material is incorporated into, onto or is formed into an article of manufacture, such as surgical sponge or as part of a microbead.

- 5           It is understood that the precise components and optimal means for application or storage are a function of the selected bioluminescence system. The concentrations of the components, which can be determined empirically, are not critical, but must be sufficient to produce a visible glow when combined. Typical concentrations are as low as nanomoles/l, preferably on the
- 10           order of mg/l or higher. The concentration on the substrate is that produced when a composition containing such typical concentration is applied to the material. Again, such ideal concentrations can be readily determined empirically by applying the first composition, letting it dry, spraying the second composition, and observing the result.
- 15           The matrix material substrates contemplated herein are generally insoluble materials used to immobilize ligands and other molecules, and are those that used in many chemical syntheses and separations. Such matrices are fabricated preferably from biocompatible, more preferably from
- 20           biodegradable materials. Such substrates, also called matrices, are used, for example, in affinity chromatography, in the immobilization of biologically active materials, and during chemical syntheses of biomolecules, including proteins, amino acids and other organic molecules and polymers. The preparation of and use of matrices is well known to those of skill in this art; there are many such materials and preparations thereof known. For example, naturally-occurring
- 25           matrix materials, such as agarose and cellulose, may be isolated from their respective sources, and processed according to known protocols, and synthetic materials may be prepared in accord with known protocols. Other matrices for use herein may comprise proteins, for example carrier molecules, such as albumin.

-130-

The substrate matrices are typically insoluble materials that are solid, porous, deformable, or hard, and have any required structure and geometry, including, but not limited to: beads, pellets, disks, capillaries, hollow fibers, needles, solid fibers, random shapes, thin films and membranes. Thus, the item  
5 may be fabricated from the matrix material or combined with it, such by coating all or part of the surface or impregnating particles.

Typically, when the matrix is particulate, the particles are at least about 10-2000  $\mu\text{M}$ , but may be smaller or larger, depending upon the selected application. Selection of the matrices will be governed, at least in part, by their  
10 physical and chemical properties, such as solubility, functional groups, mechanical stability, surface area swelling propensity, hydrophobic or hydrophilic properties and intended use. For use herein, the matrices are preferably biocompatible, more preferably biodegradable matrices.

If necessary the support matrix material can be treated to contain an  
15 appropriate reactive moiety or in some cases the may be obtained commercially already containing the reactive moiety, and may thereby serve as the matrix support upon which molecules are linked. Materials containing reactive surface moieties such as amino silane linkages, hydroxyl linkages or carboxysilane linkages may be produced by well established surface chemistry techniques  
20 involving silanization reactions, or the like. Examples of these materials are those having surface silicon oxide moieties, covalently linked to gamma-amino-propylsilane, and other organic moieties; N-[3-(triethoxysilyl)propyl]phthelamic acid; and bis-(2-hydroxyethyl)aminopropyltriethoxysilane. Exemplary of readily available materials containing amino group reactive functionalities, include, but  
25 are not limited to, para-aminophenyltriethoxysilane. Also derivatized polystyrenes and other such polymers are well known and readily available to those of skill in this art [e.g., the Tentagel® Resins are available with a multitude of functional groups, and are sold by Rapp Polymere, Tubingen, Germany; see, U.S. Patent No. 4,908,405 and U.S. Patent No. 5,292,814; see, also Butz et al.  
30 (1994) Peptide Res. 7:20-23; Kleine et al. (1994) Immunobiol. 190:53-66].

These matrix materials include any material that can act as a support matrix for attachment of the molecules of interest. Such materials are known



-131-

to those of skill in this art, and include those that are used as a support matrix. These materials include, but are not limited to, inorganic, natural polymers, and synthetic polymers, including, but are not limited to: cellulose, cellulose derivatives, acrylic resins, glass, silica gels, polystyrene, gelatin, polyvinyl pyrrolidone, co-polymers of vinyl and acrylamide, polystyrene cross-linked with divinylbenzene or the like [see, Merrifield (1964) *Biochemistry* 3:1385-1390], polyacrylamides, latex gels, polystyrene, dextran, polyacrylamides, rubber, silicon, plastics, nitrocellulose, celluloses, natural sponges. Of particular interest herein, are highly porous glasses [see, e.g., U.S. Patent No. 4,244,721] and others prepared by mixing a borosilicate, alcohol and water.

Synthetic matrices include, but are not limited to: acrylamides, dextran-derivatives and dextran co-polymers, agarose-polyacrylamide blends, other polymers and co-polymers with various functional groups, methacrylate derivatives and co-polymers, polystyrene and polystyrene copolymers [see, e.g., Merrifield (1964) *Biochemistry* 3:1385-1390; Berg et al. (1990) in *Innovation Perspect. Solid Phase Synth. Collect. Pap.*, Int. Symp., 1st, Epton, Roger (Ed), pp. 453-459; Berg et al. (1989) in *Pept., Proc. Eur. Pept. Symp.*, 20th, Jung, G. et al. (Eds), pp. 196-198; Berg et al. (1989) *J. Am. Chem. Soc.* 111:8024-8026; Kent et al. (1979) *Isr. J. Chem.* 17:243-247; Kent et al. (1978) *J. Org. Chem.* 43:2845-2852; Mitchell et al. (1976) *Tetrahedron Lett.* 42:3795-3798; U.S. Patent No. 4,507,230; U.S. Patent No. 4,006,117; and U.S. Patent No. 5,389,449]. Methods for preparation of such matrices are well-known to those of skill in this art.

Synthetic matrices include those made from polymers and co-polymers such as polyvinylalcohols, acrylates and acrylic acids such as polyethylene-co-acrylic acid, polyethylene-co-methacrylic acid, polyethylene-co-ethylacrylate, polyethylene-co-methyl acrylate, polypropylene-co-acrylic acid, polypropylene-co-methyl-acrylic acid, polypropylene-co-ethylacrylate, polypropylene-co-methyl acrylate, polyethylene-co-vinyl acetate, polypropylene-co-vinyl acetate, and those containing acid anhydride groups such as polyethylene-co-maleic anhydride, polypropylene-co-maleic anhydride and the

-132-

like. Liposomes have also been used as solid supports for affinity purifications [Powell *et al.* (1989) *Biotechnol. Bioeng.* 33:173].

- For example, U.S. Patent No. 5,403,750, describes the preparation of polyurethane-based polymers. U.S. Pat. No. 4,241,537 describes a plant
- 5 growth medium containing a hydrophilic polyurethane gel composition prepared from chain-extended polyols; random copolymerization is preferred with up to 50% propylene oxide units so that the prepolymer will be a liquid at room temperature. U.S. Pat. No. 3,939,123 describes lightly crosslinked polyurethane polymers of isocyanate terminated prepolymers containing poly(ethyleneoxy)
- 10 glycols with up to 35% of a poly(propyleneoxy) glycol or a poly(butyleneoxy) glycol. In producing these polymers, an organic polyamine is used as a crosslinking agent. Other matrices and preparation thereof are described in U.S. Patent Nos. 4,177,038, 4,175,183, 4,439,585, 4,485,227, 4,569,981, 5,092,992, 5,334,640, 5,328,603
- 15 U.S. Patent No. 4,162,355 describes a polymer suitable for use in affinity chromatography, which is a polymer of an aminimide and a vinyl compound having at least one pendant halo-methyl group. An amine ligand, which affords sites for binding in affinity chromatography is coupled to the polymer by reaction with a portion of the pendant halo-methyl groups and the
- 20 remainder of the pendant halo-methyl groups are reacted with an amine containing a pendant hydrophilic group. A method of coating a substrate with this polymer is also described. An exemplary aminimide is 1,1-dimethyl-1-(2-hydroxyoctyl)amine methacrylimide and vinyl compound is a chloromethyl styrene.
- 25 U.S. Patent No. 4,171,412 describes specific matrices based on hydrophilic polymeric gels, preferably of a macroporous character, which carry covalently bonded D-amino acids or peptides that contain D-amino acid units. The basic support is prepared by copolymerization of hydroxyalkyl esters or hydroxyalkylamides of acrylic and methacrylic acid with crosslinking acrylate or
- 30 methacrylate comonomers are modified by the reaction with diamines, aminoacids or dicarboxylic acids and the resulting carboxyterminal or aminoterminal groups are condensed with D-analogs of aminoacids or peptides.

-133-

The peptide containing D-aminoacids also can be synthesized stepwise on the surface of the carrier.

U.S. Patent No. 4,178,439 describes a cationic ion exchanger and a method for preparation thereof. U.S. Patent No. 4,180,524 describes chemical  
5 syntheses on a silica support.

Immobilized Artificial Membranes (IAMs; see, e.g., U.S. Patent Nos. 4,931,498 and 4,927,879) may also be used. IAMs mimic cell membrane environments and may be used to bind molecules that preferentially associate with cell membranes [see, e.g., Pidgeon et al. (1990) Enzyme Microb. Technol.  
10 12:149].

These materials are also used for preparing articles of manufacture, surgical sponges soaps, and other items, and thus are amenable to linkage of molecules, either the luciferase, luciferin, mixtures of both. For example, matrix particles may be impregnated into items that will then be  
15 contacted with an activator.

Kits containing the item including the matrix material with or without the coating of the GFPs or bioluminescence generating components, and compositions containing the remaining components are provided.

**b. Immobilization and activation**

20 Numerous methods have been developed for the immobilization of proteins and other biomolecules onto insoluble or liquid supports [see, e.g., Mosbach (1976) Methods in Enzymology 44; Weetall (1975) Immobilized Enzymes, Antigens, Antibodies, and Peptides; and Kennedy et al. (1983) Solid Phase Biochemistry, Analytical and Synthetic Aspects, Scouten, ed., pp.  
25 253-391; see, generally, Affinity Techniques. Enzyme Purification: Part B. Methods in Enzymology, Vol. 34, ed. W. B. Jakoby, M. Wilchek, Acad. Press, N.Y. (1974); Immobilized Biochemicals and Affinity Chromatography, Advances in Experimental Medicine and Biology, vol. 42, ed. R. Dunlap, Plenum Press, N.Y. (1974)].

30 Among the most commonly used methods are absorption and adsorption or covalent binding to the support, either directly or via a linker, such as the numerous disulfide linkages, thioether bonds, hindered disulfide bonds, and

-134-

covalent bonds between free reactive groups, such as amine and thiol groups, known to those of skill in art [see, e.g., the PIERCE CATALOG, ImmunoTechnology Catalog & Handbook, 1992-1993, which describes the preparation of and use of such reagents and provides a commercial source for such reagents; and Wong (1993) Chemistry of Protein Conjugation and Cross Linking, CRC Press; see, also DeWitt et al. (1993) Proc. Natl. Acad. Sci. U.S.A. 90:6909; Zuckermann et al. (1992) J. Am. Chem. Soc. 114:10646; Kurth et al. (1994) J. Am. Chem. Soc. 116:2661; Ellman et al. (1994) Proc. Natl. Acad. Sci. U.S.A. 91:4708; Sucholeiki (1994) Tetrahedron Ltrrs. 35:7307; and Sun Wang (1976) J. Org. Chem. 41:3258; Padwa et al. (1971) J. Org. Chem. 41:3550 and Vedejs et al. (1984) J. Org. Chem. 49:575, which describe photosensitive linkers]

To effect immobilization, a solution of the protein or other biomolecule is contacted with a support material such as alumina, carbon, an ion-exchange resin, cellulose, glass or a ceramic. Fluorocarbon polymers have been used as supports to which biomolecules have been attached by adsorption [see, U.S. Pat. No. 3,843,443; Published International PCT Application WO/86 03840]. For purposes herein, the support material will be biocompatible (i.e., suitable for use in the body).

A large variety of methods are known for attaching biological molecules, including proteins and nucleic acids, molecules to solid supports [see, e.g., U.S. Patent No. 5451683]. For example, U.S. Pat. No. 4,681,870 describes a method for introducing free amino or carboxyl groups onto a silica matrix. These groups may subsequently be covalently linked to other groups, such as a protein or other anti-ligand, in the presence of a carbodiimide. Alternatively, a silica matrix may be activated by treatment with a cyanogen halide under alkaline conditions. The anti-ligand is covalently attached to the surface upon addition to the activated surface. Another method involves modification of a polymer surface through the successive application of multiple layers of biotin, avidin and extenders [see, e.g., U.S. Patent No. 4,282,287]; other methods involve photoactivation in which a polypeptide chain is attached to a solid substrate by incorporating a light-sensitive unnatural amino acid group into the

-135-

polypeptide chain and exposing the product to low-energy ultraviolet light [see, e.g., U.S. Patent No. 4,762,881]. Oligonucleotides have also been attached using a photochemically active reagents, such as a psoralen compound, and a coupling agent, which attaches the photoreagent to the substrate [see, e.g., U.S. Patent No. 4,542,102 and U.S. Patent No. 4,562,157]. Photoactivation of the photoreagent binds a nucleic acid molecule to the substrate to give a surface-bound probe.

Covalent binding of the protein or other biomolecule or organic molecule or biological particle to chemically activated solid matrix supports such as glass, synthetic polymers, and cross-linked polysaccharides is a more frequently used immobilization technique. The molecule or biological particle may be directly linked to the matrix support or linked via linker, such as a metal [see, e.g., U.S. Patent No. 4,179,402; and Smith et al. (1992) Methods: A Companion to Methods in Enz. 4:73-78]. An example of this method is the cyanogen bromide activation of polysaccharide supports, such as agarose. The use of perfluorocarbon polymer-based supports for enzyme immobilization and affinity chromatography is described in U.S. Pat. No. 4,885,250]. In this method the biomolecule is first modified by reaction with a perfluoroalkylating agent such as perfluorooctylpropylisocyanate described in U.S. Pat. No. 4,954,444. Then, the modified protein is adsorbed onto the fluorocarbon support to effect immobilization.

The activation and use of matrices are well known and may be effected by any such known methods [see, e.g., Hermanson et al. (1992) Immobilized Affinity Ligand Techniques, Academic Press, Inc., San Diego]. For example, the coupling of the amino acids may be accomplished by techniques familiar to those in the art and provided, for example, in Stewart and Young, 1984, Solid Phase Synthesis, Second Edition, Pierce Chemical Co., Rockford.

Other suitable methods for linking molecules to solid supports are well known to those of skill in this art [see, e.g., U.S. Patent No. 5,416,193]. These include linkers that are suitable for chemically linking molecules, such as proteins, to supports and include, but are not limited to, disulfide bonds, thioether bonds, hindered disulfide bonds, and covalent bonds between free

-136-

reactive groups, such as amine and thiol groups. These bonds can be produced using heterobifunctional reagents to produce reactive thiol groups on one or both of the moieties and then reacting the thiol groups on one moiety with reactive thiol groups or amine groups to which reactive maleimido groups or

5 thiol groups can be attached on the other. Other linkers include, acid cleavable linkers, such as bismaleimideoxy propane, acid labile-transferrin conjugates and adipic acid dihydrazide, that would be cleaved in more acidic intracellular compartments; cross linkers that are cleaved upon exposure to UV or visible light and linkers, such as the various domains, such as C<sub>H</sub>1, C<sub>H</sub>2, and C<sub>H</sub>3, from

10 the constant region of human IgG<sub>1</sub> (see, Batra et al. (1993) Molecular Immunol. 30:379-386). Presently preferred linkages are direct linkages effected by adsorbing the molecule to the surface of the matrix. Other linkages are photocleavable linkages that can be activated by exposure to light [see, e.g., Goldmacher et al. (1992) Bioconj. Chem. 3:104-107, which linkers are herein

15 incorporated by reference]. The photocleavable linker is selected such that the cleaving wavelength that does not damage linked moieties. Photocleavable linkers are linkers that are cleaved upon exposure to light [see, e.g., Hazum et al. (1981) in Pept., Proc. Eur. Pept. Symp., 16th, Brunfeldt, K (Ed), pp. 105-110, which describes the use of a nitrobenzyl group as a photocleavable

20 protective group for cysteine; Yen et al. (1989) Makromol. Chem 190:69-82, which describes water soluble photocleavable copolymers, including hydroxypropylmethacrylamide copolymer, glycine copolymer, fluorescein copolymer and methylrhodamine copolymer; Goldmacher et al. (1992) Bioconj. Chem. 3:104-107, which describes a cross-linker and reagent that undergoes

25 photolytic degradation upon exposure to near UV light (350 nm); and Senter et al. (1985) Photochem. Photobiol 42:231-237, which describes nitrobenzyloxycarbonyl chloride cross linking reagents that produce photocleavable linkages]. The selected linker will depend upon the particular application and, if needed, may be empirically selected.

-137-

These methods for linking molecules to supports may be adapted for use to link the targeting agents to the targeted agents.

**M. Bioluminescence Resonance Energy Transfer (BRET) System**

- In nature, coelenterazine-using luciferases emit broadband blue-green light (max. ~480 nm). Bioluminescence Resonance Energy Transfer (BRET) is a natural phenomenon first inferred from studies of the hydrozoan *Obelia* (Morin & Hastings (1971) J. Cell Physiol. 77:313-18), whereby the green bioluminescent emission observed *in vivo* was shown to be the result of the luciferase non-radiatively transferring energy to an accessory green fluorescent protein (GFP).
- BRET was soon thereafter observed in the hydrozoan *Aequorea victoria* and the anthozoan *Renilla reniformis*. Although energy transfer *in vitro* between purified luciferase and GFP has been demonstrated in *Aequorea* (Morise *et al.* (1974) Biochemistry 13: 2656-62) and *Renilla* (Ward & Cormier (1976) J. Phys. Chem. 80:2289-91) systems, a key difference is that in solution efficient radiationless energy transfer occurs only in *Renilla*, apparently due to the pre-association of one luciferase molecule with one GFP homodimer (Ward & Cormier (1978) Photochem. Photobiol. 27:389-96). The blue (486 nm) luminescent emission of *Renilla* luciferase can be completely converted to narrow band green emission (508 nm) upon addition of proper amounts of *Renilla* GFP (Ward & Cormier (1976) J. Phys. Chem. 80: 2289-91). GFPs accept energy from excited states of luciferase-substrate complexes and re-emit the light as narrow-band green light (~510 nm). By virtue of the non-radiative energy transfer, the quantum yield of the luciferase is increased.

- Luciferases and fluorescent proteins have many well-developed and valuable uses as protein tags and transcriptional reporters; BRET has the potential to increase the sensitivity and scope of these applications. A GFP increases the sensitivity of the luciferase reporter by raising the quantum yield. A single luciferase fused to several spectrally distinct GFPs allows the simultaneous use of multiple luciferase reporters, activated by addition of a single luciferin. By preparing two fusion proteins, each containing a GFP having a different emission wavelength fused to identical luciferases, two or more reporters can be used with a single substrate addition. Thus multiple events

-138-

may be monitored or multiple assays run using a single reagent addition. Such a reporter system is self-ratioing if the distribution of luciferin is uniform or reproducible.

The ability to conveniently monitor several simultaneous macromolecular  
5 events within a cell is a major improvement over current bioluminescent technology. BRET also enables completely new modes of reporting by exploiting changes in association or orientation of the luciferase and fluorescent protein. By making fusion proteins, the luciferase-GFP acceptor pair may be made to respond to changes in association or conformation of the fused  
10 moieties and hence serves as a sensor.

Energy transfer between two fluorescent proteins (FRET) as a physiological reporter has been reported [Miyawaki et al. (1997) Nature 388:882-7], in which two different GFPs were fused to the carboxyl and amino termini of calmodulin. Changes in calcium ion concentration caused a sufficient  
15 conformational change in calmodulin to alter the level of energy transfer between the GFP moieties. The observed change in donor emission was ~10% while the change in ratio was ~1.8.

The similar use of a luciferase-GFP pair in the presence of substrate luciferin as provided herein has important advantages. First, there is no  
20 background and no excitation of the acceptor from the primary exciting light. Second, because the quantum yield of the luciferase is greatly enhanced by nonradiative transfer to GFP, background from donor emission is less, and the signal from the acceptor relatively greater. Third, the wavelength shift from the peak emission of luciferase (~480 nm) to that of the GFP (typically 508-510  
25 nm) is large, minimizing signal overlap. All three factors combine to increase the signal-to-noise ratio. The concentration of the GFP acceptor can be independently ascertained by using fluorescence.

For some applications, *in vitro* crosslinked or otherwise *in vitro* modified versions of the native proteins is contemplated. The genetically encoded fusion  
30 proteins have many great advantages: A) In vivo use - unlike chemistry-based luminescence or radioactivity-based assays, fusion proteins can be genetically incorporated into living cells or whole organisms. This greatly increases the



-139-

range of possible applications; B) Flexible and precise modification - many different response modifying elements can be reproducibly and quantitatively incorporated into a given luciferase-GFP pair; C) Simple purification - only one reagent would need to be purified, and its purification could be monitored via the fluorescent protein moiety. Ligand-binding motifs can be incorporated to facilitate affinity purification methods.

#### 1. Design of sensors based on BRET

Resonance energy transfer between two chromophores is a quantum mechanical process that is exquisitely sensitive to the distance between the donor and acceptor chromophores and their relative orientation in space (Wu & Brand (1994) Anal. Biochem. 218 1-13). Efficiency of energy transfer is inversely proportional to the 6<sup>th</sup> power of chromophore separation. In practice, the useful distance range is about 10 to 100 Å, which has made resonance energy transfer a very useful technique for studying the interactions of biological macromolecules. A variety of fluorescence-based FRET biosensors have been constructed, initially employing chemical fluors conjugated to proteins or membrane components, and more recently, using pairs of spectrally distinct GFP mutants (Giuliano & Taylor (1998) Trends Biotech. 16: 99-146; Tsien (1998) Annu. Rev. Biochem. 67:509-44).

Although these genetically encoded GFP bioluminescence -based biosensors have advantages over less convenient and less precise chemical conjugate-based biosensors, all share a limitation in their design: it is generally difficult to construct a biosensor in which energy transfer is quantitative when the chromophores are in closest apposition. It is almost impossible to arbitrarily manipulate the complex stereochemistry of proteins so that conjugated or intrinsic chromophores are *stably positioned with minimal separation and optimal orientation*. The efficiency of such biosensors are also often limited by stoichiometric imbalances between resonance energy donor and acceptor; the donor and acceptor macromolecules must be quantitatively complexed to avoid background signal emanating from uncomplexed chromophores. These limitations in general design become important when biosensors must be robust, convenient and cheap. Developing technologies such as high throughput

-140-

screening for candidate drugs (using high throughput screening (HTS) protocols), biochips and environmental monitoring systems would benefit greatly from modular biosensors where the signal of a rare target "hit" (e.g., complex formation between two polypeptides) is unambiguously (statistically) distinguishable from the huge excess of "non-hits"). Current genetically encoded FRET and bioluminescence-based biosensors display hit signals that very often are less than two-fold greater than non-hit signals, and are at best a few-fold greater (Xu et al. (1999) Proc. Natl. Acad. Sci USA 96: 151-156; Miyawaki et al. (1997) Nature 388:882-7).

To solve these problems, the anthozoan GFPs, such as the *Renilla* GFPs, provided herein can be used in combination with their cognate luciferases. Anthozoan luciferases-GFP complexes provide a "scaffold" upon which protein domains that confer the biological properties specific to a given biosensor can be linked. Although one can construct many useful two component biosensors based on this scaffold, in a biosensor contemplated herein, independent protein domains that potentially complex with one another are respectively fused to the luciferase and the GFP.

In isolation, an anthozoan luciferase emits blue light from the coelenterazine-derived chromophore (A), and an anthozoan GFP that is excited with blue-green light emits green light from its integral peptide based fluorophore (B). When the luciferase and GFP associate as a complex *in vivo* or *in vitro*, the luciferase non-radiatively transfers its reaction energy to the GFP fluorophore, which then emits the green (C). Thus any molecular interaction that disrupts the luciferase-GFP complex can be quantitatively monitored by observing the ratio of blue to green light (D).

There are many possible variations on this theme. For example, in a three component system either the luciferase or GFP can be fused to a ligand-binding domain from a protein of interest or other target peptide or other moiety of interest. If the design of the fusion protein is correct, binding of a small molecule or protein ligand then prevents the luciferase-GFP association, and one has a BRET-based biosensor. More complex protein fusions can be designed to

-141-

create two component and even single component BRET biosensors for a multitude of uses.

FIGURE 11 illustrates the underlying principle of Bioluminescent Resonance Energy Transfer (BRET) and its use as sensor: A) in isolation, a  
5 luciferase, preferably an anthozoan luciferase, emits blue light from the coelenterazine-derived chromophore; B) in isolation, a GFP, preferably an anthozoan GFP that binds to the luciferase, that is excited with blue-green light emits green light from its integral peptide based fluorphore; C) when the luciferase and GFP associate as a complex *in vivo* or *in vitro*, the luciferase non-  
10 radiatively transfers its reaction energy to the GFP fluorphore, which then emits the green light; D) any molecular interaction that disrupts the luciferase-GFP complex can be quantitatively monitored by observing the spectral shift from green to blue light.

15 The nucleic acids, and the constructs and plasmids herein, permit preparation of a variety of configurations of fusion proteins that include an anthozoan GFP, such as *Renilla*, with its cognate anthozoan luciferase. The nucleic acid encoding the GFP can be fused adjacent to the nucleic acid encoding the luciferase or separated therefrom by insertion of nucleic acid  
20 encoding, for example, a ligand-binding domain of a protein of interest. The GFP and luciferase will be bound. Upon interaction of the ligand-binding domain with the a test compound or other moiety, the interaction of the GFP and luciferase will be altered thereby changing the emission signal of the complex. If necessary the GFP and luciferase can be modified to fine tune the interaction  
25 to make it more sensitive to conformational changes or to temperature or other parameters.

## 2. Advantages of BRET sensors

There are many advantages to the BRET sensors provided herein. For example, BRET sensors are self-ratioging. The reporter and target are integrated  
30 into single polypeptide. This ensures 1:1:1 stoichiometry among luciferase, GFP and target (or a 1:N:1 stoichiometry if more than one, typically a homodimer, GFP can be bound to a luciferase). GFP fluorescence allows

-142-

absolute quantitation of sensor. The null state gives signal that verifies sensor functionality. Quantifiable null state facilitates disruption-of-BRET sensors (DBRET). BRET sensors have better signal-to-noise ratio than GFP FRET sensors because there is no cellular autofluorescence, no excitation of the  
5 acceptor from the primary exciting light, the quantum yield of luciferase greatly enhanced by non-radiative energy transfer to GFP, and there is minimal signal overlap between emission of the luciferase and emission of the GFP. Also, anthozoan GFPs have 6-fold higher extinction coefficients than Aequorea GFP.

The BRET sensors can for used for hit identification and downstream  
10 evaluation in *in vitro* screening assays in *in vitro* or *in vivo* or *in situ*, including in cultured cells and tissues and animals. The BRET sensors can be created by thermal endpoint-selection, which is suited to DBRET (Disruption-of-BRET) and reduces need for knowledge of target 3D structure and functional dynamics. Existing screening robotics to optimize  
15 biosensors. BRET sensors benefit from vast genetic diversity anthozoans have evolved efficient luciferase-GFP energy transfer systems and the components can be mixed and matched. Highly efficient heterologous luciferases may be substituted for less active luciferases. For example, a copepod luciferase active site can be fused to an anthozoan luciferase GFP-  
20 binding domain. There are many diverse coelenterazine-using luciferases.

BRET sensors are modular so that an optimized sensor scaffold may be used with different targets. Also the BRET acceptor may be varied to give shifted emissions, facilitating multiple simultaneous readouts. The anthozoan GFPs can be mutated, GFPs or other proteins can be modified with different  
25 chemical fluors, high throughput screening (HTS) fluor-modified FRET acceptors can be adapted, the BRET donor (luciferase) may be varied, such as by using an Aequorin (Ca<sup>++</sup> activated) photoprotein, or a firefly luciferase (requires ATP and a firefly luciferin) to give conditional activation. The sensor scaffold can be incorporated into a variety of immobilization motifs, including free format plates,  
30 which can reduce reagent volumes, reusable microtiter plates, miniature columns

-143-

and biochips. Finally, BRET sensors are inexpensive and reproducible reagents because they can be produced by standardized protein production and can incorporate purification tags. Genetically encoded reporters more reproducible than chemically modified reporters. Linear translation of BRET modules ensures  
5 sensor integrity.

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

#### EXAMPLE 1

##### 10 I. TOXICOLOGY

###### 1. Solubility of Coelenterazine

Coelenterazine is not terribly soluble in non-irritant vehicles.

Coelenterazine is soluble to at least to a concentration of 200 micrograms/mL in a solution of 2% (w/v) PEG 400 containing about 0.8% (w/v) NaCl. Although  
15 this solution is slightly hypertonic, it is not an irritant for vehicle purposes.

###### 2. Toxicology of Coelenterazine

###### A. Topical Administration

To examine the toxicology of the above-described coelenterazine solution, the solution was administered in the eyes of anesthetized rabbits  
20 following standard procedures and conjunctival irritation was measured. Animals were sedated with diazepam (about 2 mg/kg) and 100  $\mu$ L of the coelenterazine in the PEG solution was instilled in one eye and to the other eye only the PEG vehicle was instilled. Animals were observed for a 30-min period and then the animals were carefully examined for any conjunctival irritation as  
25 well as any corneal ulceration. The examination was performed using a slit-lamp to visualize the eye well. Only minimal conjunctival irritation from the vehicle was observed in either eye (n=3). Thus, the direct administration to the eye of about 20  $\mu$ g of coelenterazine in this solution produced no irritation, ulceration, or other signs of toxicity in this topical assay.

###### B. Intravenous Administration

30 In a second experiment, mice are administered coelenterazine (n=6) at a concentration 1 mg/kg, i.p. or vehicle (n=6) for a seven-day period. Mice are

-144-

examined over the course of the study for any gross signs of toxicity as evidenced in their behavior.

At the end of the one-week period, blood is collected by cardiac puncture immediately prior to sacrifice. Animals are sacrificed and ten different  
5 tissue samples are removed post mortem from each animal. Isolated tissues are fixed, stained, blocked and sectioned. The pathology of the tissue samples are analyzed and the toxicology data are compiled. Daily administration of coelenterazine for three days resulted in no gross behavior changes in the test animals.

10 C. Stability of Coelenterazine

The stability of coelenterazine may be determined by analyzing biological samples for the presence of coelenterazine and metabolic products derived therefrom. In this experiment, blood will be collected and serum prepared, and this serum can be assayed for coelenterazine and its metabolites. Little  
15 interference was observed from the serum (mouse) at the emission wavelength requisite for coelenterazine.

Alternatively, a lobe of liver may be resected from each animal and separately pooled, fixed, homogenized in cold acid acetone, and assayed for coelenterazine and its metabolites by standard biochemical analyses.

20 D. Coelenterazine Assays

The concentration of coelenterazine may be determined using its inherent fluorescence properties. For example, coelenterazine may be measured in an alcohol solution by measuring the fluorescence at a specified wavelength. To date, the detectable limit is less than 10 ng/mL. Given the dosages  
25 contemplated herein, this level of sensitivity should be sufficient for accurate measurement.

The concentration of coelenterazine may also be determined by use of HPLC in combination with fluorescence detection. In addition to an HPLC-based detection system, coelenterazine and its metabolites may be identified by Gas  
30 Chromatography (GC) or by Mass Spectrophotometry analysis. Final confirmation of the identity of coelenterazine and its metabolites may be performed by nuclear magnetic resonance (NMR).

-145-

### Method of Preparing Photoprotein Conjugates

A method for the preparation of photoprotein conjugates that retain bioluminescent activity has been described (see U.S. Patent No. 5,486,455). In general, additional sulfhydryl groups are introduced into the photoprotein by  
5 treatment of the photoprotein with Trauts Reagent (2-iminothiolane) to generate a sulfhydryl-activated photoprotein. The sulfhydryl-activated photoprotein is conjugated to a sulfhydryl-reactive binding reagent (e.g., a macromolecule that has been chemically modified with a heterobifunctional linker that is capable of  
10 sulfhydryl crosslinking, such as maleimido- or sulfo-SMCC, sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate). The conjugated photoprotein may be used in crude form or may be further purified by methods known to those of skill in the art, such as ion exchange or affinity chromatography.

### EXAMPLE 2

#### Rodent Model

15 A monoclonal antibody directed against a human tumor antigen (e.g., Lewis antigen or carcinoembryonic antigen [CEA]) or a humanized derivative thereof is conjugated to a photoprotein, preferably aequorin, or to the vargula luciferase, via the sulfhydryl-binding method (see U.S. Patent No. 5,486,455) and the conjugate is purified. Approximately, 10-100 micrograms of the  
20 antibody-photoprotein conjugate is injected i.v. in the tail vein of a transgenic mouse which expresses a human tumor antigen. The injection should be tolerated well by the animal.

After sufficient time is allowed for antibody binding (2-48 hours), approximately 1  $\mu$ g of the coelenterazine or 10  $\mu$ L of crude lysate containing the  
25 remaining bioluminescence generating components is injected i.p. directly in the region of the proposed neoplasm. Alternatively, 10  $\mu$ L of the lysate or 1  $\mu$ g of coelenterazine is i.p. injected and time allowed for the coelenterazine to circulate to the target region (25 minutes to 2 days).

The mouse is then anesthetized and the region containing the neoplasm  
30 is exposed in a dark room. Regions that emit light as determined by a photometer or by the human eye are targeted for surgical removal. Alternatively, the region of interest may be visualized by the insertion of a

-146-

laparoscope near the site of the neoplasm and subsequent placement of the imaging camera in a position to observe light.

### EXAMPLE 3

#### ISOLATION AND IDENTIFICATION OF DNA ENCODING *Renilla mulleri* GFP

##### 5 1. Preparation of a *R. mulleri* cDNA expression library

A *R. mulleri* cDNA expression library was prepared using the commercially available Lambda-UniZap XR Vector kit (Stratagene) according to the direction provided. Briefly, EcoRI and XhoI adaptors were ligated to 5'-end of the cDNA fragments and the ligated cDNA fragments were purified from the  
10 remaining unligated adaptors. The purified cDNAs were ligated into EcoRI- and XhoI-digested  $\lambda$  Uni-ZAP XR vector, transformed into competent *E. coli* XL-1 Blue cells and the resulting DNA was packaged into viral particles using  $\lambda$  phage helper extracts (Gigapak Plus Kit, Stratagene). The packaged lambda library was titered in *E. coli* XL-1 Blue cells and the sequence complexity of the *Renilla*  
15 *mulleri* cDNA expression library was calculated to be about  $1.73 \times 10^6$  independent plaques.

A plasmid library was derived from the lambda cDNA expression library by excision of the initiator-terminator cassette harboring the cloned *Renilla* cDNA. Approximately  $2 \times 10^8$  independent plaque isolates were pooled and  
20 used to infect *E. coli* SOLR cells (Stratagene), which were then co-infected with a filamentous helper phage VCSM13, R408 or ExAssist helper phage (Stratagene). The cDNA-containing plasmids were recovered by plating the infected cells on solid medium supplemented with 200  $\mu$ g/ml ampicillin for the selection of cells containing excised pBK plasmid.

25 In *E. coli* XL-1 Blue cells, the expression of the *Renilla mulleri* GFP in the pBK plasmid is under the control of the lacZ promoter, whose transcription is easily induced by the addition of isopropylthio- $\beta$ -D-galactopyranoside (IPTG) to the culture medium or may be applied directly to the colonies in spray form or other aerosols.

##### 30 2. cDNA library screening

To identify clones expressing a *Renilla* GFP, a functional screening method employing blue light, e.g., 490 nm, was used to identify fluorescent



-147-

GFP transformants expressing a *Renilla* GFP. The *Renilla* cDNA expression plasmid library was screened by transforming competent *E. coli* XL-1 Blue cells and plating a portion of the transformation mixture on L-broth plates supplemented with 200  $\mu$ g/ml ampicillin containing carbon black, which was added to completely absorb background fluorescence (e.g., from the agar). Plates were illuminated with narrow bandwidth light centered at 490 nm and observed through a 510 nm narrow bandpass filter using methods generally known in the art (e.g., see Ward *et al.* (1978) J. Biol. Chem. 254: 781-788).

Approximately  $3-4 \times 10^6$  individual colonies were screened and a three light emitting colony were identified. To confirm that the above-described strain harbored a plasmid that encoded a GFP, the spectral properties of the plasmid-encoded protein were assessed using cell lysates and partially purified cell extracts (e.g., see EXAMPLE 4). The fluorescence excitation spectrum for partially purified recombinant *Renilla mulleri* GFP was similar to those reported for other *Renilla* species (maximum near 498 nm); however, the recombinant *R. mulleri* GFP emission spectrum has a wavelength maximum near 506 nm, which is a slightly shorter wavelength maximum than the in vitro and in vivo emission spectrum obtained for naturally-occurring *Renilla* GFP (e.g., 509 nm; see Wampler *et al.* (1973) Biochem. Biophys. Acta 314:104-109).

20           3.       Determination and characterization of the nucleotide sequence of DNA encoding *Renilla mulleri* GFP

Plasmid DNA was purified from cultures of the fluorescent transformant and the nucleotide sequence of the *Renilla* cDNA plasmid insert was determined using methods well known to those of skill in the art (e.g., see Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Sanger *et al.* (1) Proc. Natl. Acad. Sci. U.S.A. ).

The nucleotide sequence of a cDNA encoding a full-length *Renilla mulleri* GFP is set forth in SEQ ID No. 15. The cDNA fragment encoding the *Renilla mulleri* GFP is 1,079 nt in length, including 258 nt of 5' noncoding sequence, a 714 nt open reading frame, encoding a 238 amino acid polypeptide, and 107 nt of 3'non-coding sequence.

-148-

The nucleotide sequence of the cDNA encoding the *Renilla* GFP was compared to the nucleotide sequence of the *A. victoria* GFP, the only other GFP whose complete nucleotide sequence is known (e.g., see SEQ ID No. 1). The nucleic acids isolated from the two organisms encode proteins of identical length, however, the nucleotide sequence that encodes the amino-terminal 136 amino acid residues of the *Renilla mulleri* GFP is only 48.8% identical compared to *A. victoria*. Furthermore, the nucleotide sequence encoding the carboxy-terminal 102 amino acid residues of *Renilla mulleri* GFP is even more highly divergent, being only 31.4% identical.

10 A comparison of the deduced amino acid sequences of the *Renilla mulleri* GFP and the *A. victoria* GFP revealed the protein sequences are also highly divergent. Only 56 of 238 amino acid residues between the deduced amino acid sequences are identical (i.e., 23.5% direct amino acid identity). Moreover, the deduced sequence of the putative hexapeptide chromophore in *R. mulleri* (FQYGNR) is quite different from that of *A. victoria* (FSYGVQ) having only 3 out of 6 identical amino acid residues. The *Renilla mulleri* chromophore is also located in a slight different position in the polypeptide chain compared to *A. victoria* GFP. The *R. mulleri* chromophore is encoded by amino acid residues 68-73 whereas the *A. victoria* chromophore is encoded by amino acid residues 64-69. The slightly different position and altered chromophore sequence likely contribute to the differing spectral properties exhibited by the two proteins.

#### EXAMPLE 4

##### 25 IDENTIFICATION AND ISOLATION OF DNA ENCODING A *Renilla mulleri* LUCIFERASE

The *R. mulleri* cDNA plasmid library described in EXAMPLE 3 was transformed in *E. coli* XL-1 Blue cells and single colonies were obtained by plating a portion of the transformation mixture on L-broth plates supplemented with 200 µg/ml ampicillin and also supplemented with carbon black to absorb background fluorescence. The plates were incubated overnight at 37°C. Ampicillin resistant transformants were sprayed with a 1 mM IPTG solution to induce luciferase expression. After allowing time for the cells to express the

-149-

luciferase, the surface of the plates were sprayed with a solution containing 20 mM coelenterazine and colonies emitting blue light were visualized using a blue bandwidth filter. Plasmid DNA was isolated from cultures of bioluminescent transformants and the nucleotide sequence of a cDNA insert of a positive clone was determined. The nucleotide sequence of DNA encoding a full-length *Renilla mulleri* luciferase and the deduced amino acid sequence are set forth in SEQ ID No. 17. The cDNA fragment encoding the *Renilla mulleri* luciferase is 1,217 nt in length, including 30 nt of 5'-non-coding region, a 933 nt open reading frame encoding a 311 amino acid polypeptide and 254 nt of 3'-non-coding sequence.

5

## EXAMPLE 5

RECOMBINANT PRODUCTION *Renilla* LUCIFERASE1. Recombinant production of *Renilla reniformis* luciferase

The phagemid pTZ18R (Pharmacia) is a multi-purpose DNA vector designed for in vitro transcriptions and useful for expression of recombinant proteins in bacterial hosts. The vector contains the bla gene, which allows for the selection of transformants by resistance to ampicillin, and a polylinker site adjacent to the lacZ' gene. The heterologous gene of interest is inserted in the polylinker and transcribed from the lac promoter by induction, for example, with isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG).

20 The DNA encoding the *Renilla reniformis* luciferase has been cloned (e.g., see U.S. Patent Nos. 5,292,658 and 5,418,155). The plasmid pTZRLuc-1 encodes the *Renilla* luciferase on a 2.2 Kbp EcoRI to SstI DNA fragment inserted in EcoRI and SstI sites of pTZ18R (plasmid construction is described U.S. Patent Nos. 5,292,658 and 5,418,155; see also Lorenz et al. (1991) Isolation and Expression of a cDNA encoding *Renilla reniformis* Luciferase, Proc. Natl. Acad. Sci. U.S.A. 88, 4438-4442). The initiation of transcription of the *Renilla* luciferase cDNA is under the control of the lacZ' promoter. E. coli strains harboring plasmid pTZRLuc-1 express *Renilla* luciferase that is functional in bioluminescence assays and retains the properties of the native enzyme (see  
25 e.g., U.S. Patent Nos. 5,292,658 and 5,418,155).

30

A derivative of pTZRLuc-1, pTZRLuc-3.6, produces approximately 7-fold higher levels of recombinant *Renilla* luciferase than pTZRLuc-1 when

-150-

transformed into the same *E. coli* host. Competent *E. coli* strain XL-1 was transformed using purified pTZRLuc-3.6 according to the instructions provided by the manufacturer (XL-1 Supercompetent cells and protocol; Stratagene, Inc., La Jolla, CA). Transfectants were selected by plating on Luria Broth (LB) plates  
5 supplemented with 100 µg/ml ampicillin.

Single ampicillin resistant colonies were grown in LB medium supplemented with 100 µg/ml ampicillin at ambient temperature using continuous shaking until cell growth reached mid-log phase (i.e., cell culture reaches an O.D.<sub>600nm</sub> = 0.6-0.8 units). Transcription from the *lac* promoter was  
10 induced by addition of 1 mM IPTG and cell culture was shaken at ambient temperature for an additional 8 hours.

Cells were harvested by centrifugation at 10,000 x g and frozen at -20° C. The cell pellet was thawed and resuspended at a 1:5 ratio (w/w) in a solution of 10 mM EDTA, pH 8.0, containing 4 mg/ml lysozyme (Sigma  
15 Chemical Corp.). The cells were placed in a 25° C water bath for 30 minutes and then transferred to ice for 1 hour. The cells were lysed by sonication at 0° C using a 1 minute pulse from an Ultrasonics, Inc. cell disrupter.

The lysed cellular debris was removed by centrifugation at 30,000 x g for 3 hours and the supernatant was decanted and retained. The pellet was  
20 resuspended at a 1:5 ratio in the above-described solutions, and the subsequent incubations, lysis and centrifugation steps were repeated. The two supernatants were combined and stored at -70° C. The resulting "clarified lysate" was employed as a source of recombinant luciferase. Alternatively, the lysate may be subjected to additional purification steps (e.g.,  
25 ion exchange chromatography or immunoaffinity chromatography) to further enrich the lysate or provide a homogeneous source of the purified enzyme (see e.g., U.S. Patent Nos. 5,292,658 and 5,418,155).

Alternatively, recombinant *Renilla mulleri* luciferase may be expressed by substituting the DNA encoding the *R. reniformis* luciferase for the DNA  
30 encoding a *R. mulleri* luciferase, such as DNA encoding the sequence of amino acids set forth in SEQ ID No. 18.

#### EXAMPLE 6

-151-

**DETECTION OF CANCER CELLS**

The luciferase-based bioluminescent detection method has broad application in the visualization and precise localization of cancer cells. In such applications, the *Renilla* GFP, *Renilla mulleri* luciferase or luciferin molecule may be conjugated to a targeting agent, such as an anti-tumor antigen antibody, which specifically recognizes certain cancer cells that express the antigen. Alternatively, the luciferase is coupled to a microcarrier and the targeting agent is conjugated to the luciferase and/or the microcarrier. The conjugate is introduced into a subject, for example, through intravenous, intraperitoneal or subcutaneous injection or through topical application or direct application during surgery using a laparoscope or trocar. Through formation of an antibody-antigen complex, the luciferase or luciferin is linked to the target cancer cells and available for interaction with luciferin substrate (if the conjugate contains luciferase) or luciferase enzyme (if the conjugate contains luciferin). Thus, the substrate or enzyme is then introduced into the subject, e.g., through injection or application, and allowed to react with the partner molecule contained in the antibody conjugate to yield the readily detectable light emission only the precise areas where the conjugate is stably present as an antibody-antigen complex.

The sensitivity and biocompatibility of this bioluminescence detection system make it possible to discover cancer in its early stages, e.g., small numbers of cancer cells, in contrast to other less sensitive methods which are able to detect cancer cells only after the neoplasm has developed to a more advanced and potentially life-threatening stage. In addition, diagnostic methods disclosed herein may be utilized in the absence of invasive surgical procedures. For example, surgical viewing devices, computer tomograms or miniature surgical viewing instruments, see above, that has been modified to detect low intensity levels of visible red and near infrared light emitted through the tissues of the patient may also be used to assist the surgeon, such as those described in co-pending, co-owned U.S. application Serial No. 08/990,103.

The bioluminescence detection system is particularly applicable in surgical procedures to remove cancerous lesions. The targeting of a *Renilla* GFP, *Renilla mulleri* luciferase and/or luciferin to, e.g., a tumor, results in the

-152-

precise delineation of the boundaries of the lesion and thereby facilitates a more exact excision and complete eradication of the cancer without removal of surrounding healthy tissue. This is of critical importance in the excision of cancerous lesions in complex, vital tissues, such as nervous tissue. The

- 5 sensitivity of the bioluminescence generating system also makes it well-suited for post-surgery evaluations and identification of metastases in which the ability to detect small numbers of any remaining cancer cells enables a more accurate assessment of the effectiveness of the procedure in eradicating the cancer.

- 10 The bioluminescence generating system finds further use in monitoring the progression and spread of cancer. Such information is invaluable in assessing the effectiveness of therapies, such as chemotherapy and radiation therapy, as well as the efficacy of drug-based therapies in treating cancer patients.

#### Detection of Cervical Cancer

- 15 A luciferase-based bioluminescence detection system can be used in the detection of cervical cancer. For example, luciferin or luciferase may be conjugated, directly or through a linker or microcarrier, to antibodies specific for cervical cancer cell antigens (e.g., see Table 3). The conjugate is then directly applied in an appropriate formulation to the cervical tissue which is then rinsed
- 20 to eliminate any unbound conjugate. The remaining components of the bioluminescent reaction, i.e., luciferin if the conjugate contains luciferase or luciferase if the conjugate contains luciferin, is then applied to the cervical tissue, along with any necessary activators, and allowed to interact with any bound conjugate. Light emission is then monitored. The light emitted may be of
- 25 any visible wavelength detectable by a human eye. If cancer cells presenting the recognized antigen are present in the tissue, those cells will glow and thereby be visualized. The bioluminescence serves to provide a more precise localization of the cancer which guides a surgeon in removal of the cancerous lesion.

-153-

### Detection of Carcinoembryonic Antigen (CEA)

A luciferase-based bioluminescence detection system can also be used in the detection of neoplastic cells presenting CEA, such as, e.g., cancerous cells present in colorectal cancers (e.g., see Table 3). In this application, the

5 luciferase or luciferin is conjugated, directly or indirectly, to an antibody specific for CEA, and detection is accomplished as described above for the detection of cervical cancer. The migration of CEA-bearing cancer cells, for instance into the wall of the colon and further into the lymphatics, may also be monitored with this detection system. The modified laparoscope that detects low intensity

10 visible light may be further employed to enhance the detection and visualization of the CEA-bearing cancer cells.

### Detection of Urinary Bladder Cancer

For detection of urinary bladder cancers, the luciferase or luciferin is conjugated to a targeting agent, e.g., an antibody that recognize antigens

15 presented on bladder cancer cells, that serve to link the conjugate to the cancerous lesions. The conjugate is introduced into the bladder, for example through a catheter, and the lesions are visualized and delineated upon subsequent introduction of the remaining components of the bioluminescent reaction into the bladder. This embodiment is particularly useful for urinary

20 cancers of the bladder, which are currently removed during surgery by transurethral burning of the tumor located in the bladder wall using an electrocautery. This technique would minimize cauterization of healthy bladder tissue, identify potential areas of metastasis and ensure complete surgical removal of the target.

25 In another embodiment, the location and margins of neoplastic bladder tissue may be defined with greater particularity by detecting the presence of the tumor with targeting agent coupled to the luciferase-bound microparticle. After administration of the target agent conjugate, the bioluminescent reaction is initiated (i.e., by addition of a luciferin and/or any activators). A secondary,

30 GFP-bound microparticle is covalently linked to a targeting agent which is directed against nearby surrounding tissue or which preferentially targets identical, non-tumorigenic tissue. The GFP conjugate is administered to the

-154-

patient. Thus, for example, the neoplastic tissue would glow emitting a blue light, e.g., using aequorin or *Renilla* luciferase-targeting agent conjugate whereas the GFP-bound surrounding tissue would absorb the blue light and emit green light thereby providing additional contrast to clearly define the margins of the tissue to be surgically removed.

#### Detection of the Spread of Migratory Cancer Cells

The infiltration of the lymphatic system by migratory cancer cells, such as from cutaneous melanomas, deep breast tumors and hepatic metastases originating in colon cancer, may be readily detected using the bioluminescence detection system. The luciferase or luciferin conjugated to a targeting agent, such as an antibody that recognizes a cancer cell antigen, complexes specifically with the cells, no matter where they are in the migratory process. The remaining bioluminescence generating components are then allowed to circulate throughout the body to interact only with the cells to which conjugate is bound. In instances in which the cancer cells have invaded the epithelial tissues at or near the surface of the skin, the conjugate and/or partner molecule may be topically applied and the resulting light emission readily detected by the human eye without invasive procedures. Additionally, a photomultiplier or surgical viewing devices may also be used to amplify the light output through the skin. In this manner, it may be possible to trace lymphatic migration of tumor cells before surgery is attempted.

#### Detection of Breast Cancer

The benefits of early detection of breast cancer, e.g., increased survivability rate and greater options for treatment, are numerous and well documented. The bioluminescence detection system provides a sensitive method to facilitate early diagnosis of breast cancers. For example, in such applications, the luciferase or luciferin may be conjugated to anti-estrogen or anti-progesterone receptor antibodies which target molecules that are greatly increased in number in breast cancer tissue as opposed to normal breast tissue. Thus, in this essentially quantitative assay system, the diagnosis depends on the level of luminescence detected, for example, in biopsied breast tissue. The



-155-

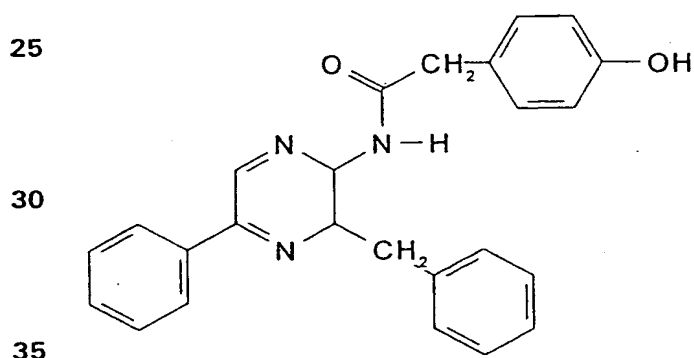
level of luminescence may be quantified using a photometer, photomultipliers or other suitable means.

Alternatively, the targeting agent may be coupled directly or indirectly to the luciferase isolated from *Aristostomias*, *Malacosteus* or *Pachystomias*, which  
5 emit red light [e.g., see Widder *et al.* (1984) *Science* 225:512-514]. Particularly preferred are bioluminescence components isolated from the species *Aristostomias scintillans* and *Malacosteus niger*. In this application, the luciferase-containing targeting agent is administered to the patient followed by  
10 the remaining components of the bioluminescence generating system (e.g., a luciferin and/or activators). Light emissions in this wavelength are detected directly through the tissue without an invasive surgical procedure using a photomultiplier, computer tomograph or using surgical vision device that is highly sensitive to red light. Alternatively, a surgical viewing instrument may be used in which the optical detector means contains a CCD imager or an image  
15 intensifier that is particularly sensitive to red light emissions.

#### EXAMPLE 7

##### AMPLIFICATION OF FLUORESCENT EMISSIONS USING *RENILLA* GFP

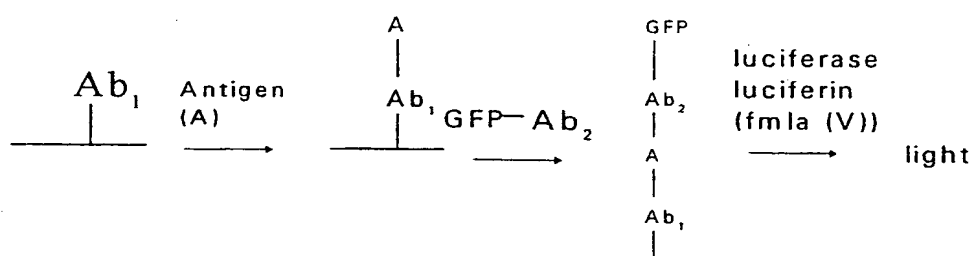
In the presence of *Renilla* GFP, the bioluminescence quantum yield of the *Renilla* luciferase-coelenterazine reaction is increased from 6.9% to 13%, an  
20 amplification of nearly two-fold. Derivatives of coelenterazine in which one or more group of the coelenterazine structure have been replaced are known (e.g., see Hart *et al.* (1979) *Biochemistry* 18:2004). Of particular interest herein is coelenterazine of formula (V), discussed above:



-156-

As noted, reaction of this compound in the presence of *Renilla* luciferase produces ultraviolet light,  $\lambda$  maximum 390 nm, and the bioluminescence quantum yield is relatively low (about 0.012%). Upon addition of GFP, however, the *Renilla* luciferase/GFP complex emits green light and the bioluminescence quantum yield is increased to 2.3%. Therefore, the addition of GFP results in an approximate 200-fold increase in the amount of light emitted by *Renilla* luciferase. Furthermore, using a bandpass filter with a exclusion limit of less than 470 nm, only those wavelengths of light greater than 470 nm can be observed. Under these conditions, the visualization of light emissions is directly dependent on the presence of a GFP in order to shift the blue photons of light to those greater than 470 nm (e.g., 510 nm green light).

The use of *Renilla* GFP in combination with a luciferase that emits blue light and this coelenterazine derivative and the bandpass filter allows for the development of immunoassays in which detectable light production is dependent upon the presence of a GFP. A number of configurations for such immunoassays are possible and an exemplary immunoassay for use in which the reaction is performed on a solid support, such as a microtiter array format is as follows:



20

When used herein, a test sample suspected of containing the target antigen(s) is added to a microtiter plate containing a plurality of antibodies specific for a targeted antigen(s) that are individually attached to the wells. After forming an antibody-antigen complex, an antibody conjugate containing a secondary antibody specific for the antigen, or  $F(Ab)_2$  fragment thereof, that is

25

-157-

linked to a *Renilla* GFP, such as those described herein is added. Specifically bound secondary antibody is detected by the addition of a luciferase, preferably a *Renilla reniformis* or *Renilla mulleri* luciferase, and the compound of formula (V) and light production is observed using a 470 nm bandpass filter. The light intensity should be a measure of the amount of GFP-Ab<sub>2</sub> present, which in turn is a measure of the amount of antigen bound to Ab<sub>1</sub>.

Using this system, the presence of the antigen in the sample is confirmed by detecting light for the individual wells to which the antibody is specific. By knowing the specificity of the antibody, the specific antigen present in the sample can be identified. Thus, it should be possible to perform immunoassays that do not require an intermediate washing step prior to the addition of luciferase/luciferin.

#### EXAMPLE 8

##### IDENTIFICATION AND ISOLATION OF DNA ENCODING A *Gaussia mulleri* LUCIFERASE

###### 1. Preparation of a *Gaussia* DNA expression library

A *Gaussia* cDNA expression library was prepared using the commercially available Lamda-UniZap XR Vector kit (Stratagene) according to the directions provided. Briefly, EcoRI and XhoI adaptors were ligated to 5'-end of the cDNA fragments and the ligated cDNA fragments were purified from the remaining unligated adaptors. The purified cDNAs were ligated into EcoRI- and XhoI-digested  $\lambda$  Uni-ZAP XR vector, transformed into competent *E. coli* XL-1 Blue cells and the resulting DNA was packaged into viral particles using  $\lambda$  phage helper extracts (Gigapak Plus Kit, Stratagene). The packaged lambda library was titered in *E. coli* XL-1 Blue cells and the sequence complexity of the cDNA expression library was calculated.

A plasmid library was derived from the lambda cDNA expression library by excision of the initiator-terminator cassette harboring the cloned *Gaussia* luciferase-encoding DNA. Approximately  $2 \times 10^8$  independent plaque isolates were pooled and used to infect *E. coli* SOLR cells (Stratagene), which were then co-infected with a filamentous helper phage VCSM13, R408 or ExAssist helper phage (Stratagene). The cDNA-containing plasmids were recovered by plating

-158-

the infected cells on solid medium supplemented with 200  $\mu$ g/ml ampicillin for the selection of cells containing excised pBK plasmid.

In *E. coli* XL-1 Blue cells, the expression of the DNA encoding the luciferase in the pBK plasmid is under the control of the *lacZ* promoter, whose transcription is easily induced by the addition of isopropylthio- $\beta$ -D-galactopyranoside (IPTG) to the culture medium or may be applied directly to the colonies in spray form or other aerosols.

## 2. cDNA library screening

To identify clones expressing a *Gaussia* luciferase, a functional screening method was used. The cDNA plasmid library transformed into *E. coli* XL-1 Blue cells and single colonies were obtained by plating a portion of the transformation mixture on L-broth plates supplemented with 200  $\mu$ g/ml ampicillin and also supplemented with carbon black to absorb background fluorescence. The plates were incubated overnight at 37°C. Ampicillin resistant transformants were sprayed with a 1 mM IPTG solution to induce luciferase expression. After allowing time for the cells to express the luciferase, the surface of the plates were sprayed with a solution containing 20 mM coelenterazine and colonies emitting blue light were visualized using a blue bandwidth filter.

Plasmid DNA was isolated from cultures of bioluminescent transformants and the nucleotide sequence of a cDNA insert of a positive clone was determined. The nucleotide sequence of DNA encoding a full-length *Gaussia* luciferase and the amino acid sequence are set forth in SEQ ID Nos. 19 and 20. The cDNA fragment encoding the *Gaussia* luciferase is 765 nt in length, including 5'-non-coding region, a 455 nt open reading frame encoding a 185 amino acid polypeptide, and 3'-non-coding sequence.

## EXAMPLE 9

### CLONING ADDITIONAL LUCIFERASE AND GFP PROTEINS

Using the methods described in the preceding examples, a nucleic acid encoding a GFP for a *Ptilosarcus* (a sea pen obtained from the Sea of Cortez) species, and a luciferase from a *Pleuromamma* (a copepod) species was obtained. The sequences are set forth in SEQ ID NOs. 28-32. The

-159-

*Pleuromamma* luciferase was cloned from a mixed copepod library of *Gaussia* and *Pleuromamma* ("giant" calenoid copepods, ~6 mm and ~3 mm, respectively). The *Pleuromamma* luciferase was identified by its greener *in vivo* emission.

- 5           Emission spectra and pH and salt curves for the encoded proteins are provided in FIGURES 4-6 and 8-10.

#### EXAMPLE 10

- Gaussia*, *Renilla mulleri* and *Pleuromamma* luciferases and *Renilla mulleri* and *Ptilosarcus* GFPs have been cloned. Various nucleic acid constructs and  
10           plasmids containing nucleic acid encoding these proteins have been prepared and have been used for expression of the encoded proteins for use in diagnostics, in analytical procedures, and in the novelty items as described herein.

#### Constructs

- 15    A.    *Renilla mulleri* luciferase and GFP-encoding constructs and plasmids  
          The host plasmid is a pBluescript SK(-) phagemid (Stratagene). The construct shown is one that was isolated by the functional screening of a large population of phagemids derived from mass excision of an amplified Lambda ZAP cDNA library (Stratagene). Each of the cloned bioluminescent genes  
20           described herein were isolated in a similar phagemid as an insert between the EcoRI and XhoI sites of the multiple cloning site (MCS). Each insert includes DNA that encompasses the entire coding region (CDS) of the functional protein, as well as a variable number of nucleotides 5' and 3' of the coding region. In addition to the amino acids of the native protein, the polypeptides expressed in  
25           the functionally screened isolates (here, the *lacZ-Renilla mulleri* luciferase cDNA fusion CDS or the *lacZ-Renilla mulleri* GFP cDNA fusion CDS) can contain additional N-terminal residues.

##### 1) *Renilla mulleri* luciferase in pBluescript SK-(r) (4147 bp)

- A *lacZ-Renilla mulleri* luciferase coding domain sequence (CDS) fusion  
30           was cloned into the pBluescript SK-(r) (Stratagene). This well known commercially available vector contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Ampicillin), a phage replication

-160-

origin (f1 origin), T3 primer sequence, T3 20-mer sequence, NIH oligo 0495 sequence, T3 promoter, SK primer sequence, T7 promoter, KS primer sequence, T7 primer sequence, T7 22-mer sequence, NIH oligo 0436 sequence, phage-plasmid PCR1 sequence, phage-plasmid PCR2 sequence, phage-plasmid

- 5 PCR2(b) sequence and various restriction cloning sites. Expression of the lacZ-*Renilla mulleri* luciferase fusion protein is under the control of lacZ promoter.

**2) *Renilla mulleri* luciferase in pBluescript SK (4147 bp)**

A lacZ-*Renilla mulleri* luciferase coding domain sequence (CDS) fusion was cloned into the pBluescript SK-(r) (Stratagene), which contains, *inter alia*, a

10 bacterial replication origin (ColE1 origin), a bacterial selectable marker (Ampicillin), a phage replication origin (f1 origin), T3 primer sequence, T3 promoter, SK primer sequence, T7 promoter, KS primer sequence, T7 primer sequence and various restriction cloning sites. Expression of the lacZ-*Renilla mulleri* luciferase fusion protein is under the control of lacZ promoter.

- 15 B. **Plasmids for expression of native *Renilla mulleri* GFP and luciferase in mammalian cells**

The *Renilla mulleri* GFP or luciferase coding region was amplified by nucleic acid amplification (PCR), respectively appending an EcoRI site and a XhoI site immediately 5' and 3' to the coding sequence. The PCR product was

20 inserted between the *EcoRI-XhoI* sites in the polylinker of pcDNA3.1(+) (Invitrogen), and transformed into bacteria (*e.g.*, XLI-Blue strain, Stratagene) for the purpose of producing large quantities of plasmid DNA. These plasmids contain the CMV promoter (Pcmv) to drive expression in mammalian cells.

**1) *Renilla mulleri* GFP in pcDNA3.1(+) (6122 bp)**

- 25 A *Renilla mulleri* GFP CDS was cloned into the pcDNA3.1(+) (Invitrogen, San Diego), which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Amp), a mammalian selectable marker (Neo), a phage replication origin (f1 origin), a viral replication origin (SV40 origin) and various restriction cloning sites. Expression of the *Renilla mulleri*
- 30 GFP is under the control of CMV promoter.

-161-

## 2) *Renilla mulleri* luciferase in pcDNA3.1(+) (6341 bp)

A *Renilla mulleri* luciferase CDS was cloned into the pcDNA3.1(+) (Invitrogen, San Diego) which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Amp), a mammalian selectable  
 5 marker (Neo), a phage replication origin (f1 origin), a viral replication origin (SV40 origin) and various restriction cloning sites. Expression of the *Renilla mulleri* luciferase is under the control of CMV promoter.

## 4. Plasmids used to express native *Renilla mulleri* GFP and luciferase in yeast cells

10 The *Renilla mulleri* GFP or luciferase was PCR amplified and inserted between the polylinker *EcoRI-XhoI* sites of pYES2 (Invitrogen). These plasmids are designed for galactose-inducible expression in yeast under regulation of the GAL1 promoter.

### 1) *Renilla mulleri* GFP in pYES2 (6547 bp)

15 A *Renilla mulleri* GFP CDS was cloned into the yeast expression plasmid pYES2 (Invitrogen, San Diego), which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Amp), a yeast replication origin (2 micron origin), a yeast selectable marker (URA3), a phage replication origin (f1 origin) and various restriction cloning sites. Expression of the *Renilla*  
 20 *mulleri* GFP is under the control of yeast GAL1. This vector is designed for expression in *Saccharomyces cerevisiae* cells.

### 2) *Renilla mulleri* luciferase in pYES2 (6766 bp)

A *Renilla mulleri* luciferase CDS was cloned into the pYES2 (Invitrogen) which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial  
 25 selectable marker (Amp), a yeast replication origin (2 micron origin), a yeast selectable marker (URA3), a phage replication origin (f1 origin) and various restriction cloning sites. Expression of the *Renilla mulleri* luciferase is under the control of yeast GAL1.

## 30 D. Plasmids used to express native *Renilla mulleri* GFP or luciferase in bacterial cells

Using the pET-34 CBD-*Renilla mulleri* luciferase or pET-34 CBD-*Renilla mulleri* GFP plasmid as template, high fidelity inverse PCR was used to -

-162-

precisely delete the CBD and all other coding sequences 5' to the native *Renilla mulleri* luciferase or GFP start codon. The plasmids were recircularized and reintroduced into BL21 (DE3) cells (Novagen, Madison, WI). These plasmids are designed to express large quantities of native-length polypeptide upon induction with IPTG. Dependent on the nature of the expressed polypeptide, the protein can fold properly and reside in a functional form in the cytosol or be released into the culture medium. When expressed in this manner, significant functional activity is observed for all bioluminescent proteins described herein. The *Gaussia* luciferase is released into the culture medium.

10           1) Native *Ptilosarcus* GFP in pET-34 (6014 bp)

A *Ptilosarcus* GFP CDS was cloned into the pET-34 (Novagen) which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Kan), a phage replication origin (f1 origin), lacI sequence, T7 promoter, lac operator, ribosome binding sequence (rbs), LIC (ligation independent cloning) site, 3' LIC overlap sequence, His tag CDS, His tag sequence, T7 terminator and various restriction cloning sites. Expression of the *Ptilosarcus* GFP is under the joint control of lac operator and T7 promoter, which is inducible by IPTG.

20           2) Native *Renilla mulleri* GFP in pET-34 (6014 bp)

A *Renilla mulleri* GFP CDS was cloned into the pET-34 (Novagen) which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Kan), a phage replication origin (f1 origin), lacI sequence, T7 promoter, lac operator, rbs, LIC site, 3' LIC overlap sequence, His tag CDS, His tag sequence, T7 terminator and various restriction cloning sites. Expression of the *Renilla mulleri* GFP is under the joint control of lac operator and T7 promoter, which is inducible by IPTG.

25           3) Native *Gaussia* luciferase in pET-34 (5855 bp)

A *Gaussia* luciferase CDS was cloned into the pET-34 (Novagen) which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Kan), a phage replication origin (f1 origin), lacI sequence, T7 promoter, lac operator, rbs, LIC site, 3' LIC overlap sequence, His tag CDS, His tag sequence, T7 terminator and various restriction cloning sites. Expression of



-163-

the *Gaussia* luciferase is under the joint control of lac operator and T7 promoter, which is inducible by IPTG.

**4) Native *Pleuromamma* luciferase in pET-34 (5894 bp)**

A *Pleuromamma* luciferase CDS was cloned into the pET-34 (Novagen) which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Kan), a phage replication origin (f1 origin), lacI sequence, T7 promoter, lac operator, rbs, LIC site, 3' LIC overlap sequence, His tag CDS, His tag sequence, T7 terminator and various restriction cloning sites. Expression of the *Pleuromamma* luciferase is under the joint control of lac operator and T7 promoter, which is inducible by IPTG.

**5) Native *Renilla mulleri* luciferase in pET-34 (6233 bp)**

A *Renilla mulleri* luciferase CDS was cloned into the pET-34 (Novagen) which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Kan), a phage replication origin (f1 origin), lacI sequence, T7 promoter, lac operator, rbs, LIC site, 3' LIC overlap sequence, His tag CDS, His tag sequence, T7 terminator and various restriction cloning sites. Expression of the *Renilla mulleri* luciferase is under the joint control of lac operator and T7 promoter, which is inducible by IPTG.

**5. Plasmids used to purify a cellulose binding domain-*Renilla mulleri* luciferase or GFP fusion protein from bacterial cells**

The coding region of the *Renilla mulleri* luciferase or GFP was amplified with a high fidelity polymerase (*Pfu turbo*, Stratagene) using the cloned pBluescript phagemid as template, and inserted using a ligation independent cloning (LIC) site into the pET-34 LIC vector (Novagen). The resulting cellulose binding domain (CBD)-luciferase or CBD-GFP fusion protein can be expressed at high levels in BLI(DE3) cells (Novagen) after induction with IPTG. Due to the nature of CBD-clos, the major portion of the expressed protein will reside in insoluble inclusion bodies. Inclusion bodies can be isolated in semi-pure state and functional CBD-fusion proteins can be recovered by renaturation. Inclusion of the thrombin and enterokinase (EK) cleavage sites in the fusion protein permits isolation of highly purified native or near-native proteins for the purposes of rigorous analysis.

-164-

**1) CBD-*Renilla mulleri* luciferase in pET-34 (6824 bp)**

A CBD-*Renilla mulleri* luciferase CDS was cloned into the pET-34 (Novagen), which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Kan), a phage replication origin (f1 origin), lacI sequence, T7 promoter, lac operator, rbs, LIC site, 3' LIC overlap sequence, His tag CDS, His tag sequence, T7 terminator, thrombin cleavage site, the RNase-S-peptide tag (S tag) CDS sequence, EK cleavage site and various restriction cloning sites. Expression of the CBD-*Renilla mulleri* luciferase is under the joint control of lac operator and T7 promoter, which is inducible by IPTG.

**10      2) CBD-*Gaussia* luciferase in pET-34 (6446 bp)**

A CBD-*Gaussia* luciferase CDS was cloned into the pET-34 (Novagen), which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Kan), a phage replication origin (f1 origin), lacI sequence, T7 promoter, lac operator, rbs, LIC site, 3' LIC overlap sequence, His tag CDS, His tag sequence, T7 terminator, thrombin cleavage site, S tag CDS sequence, EK cleavage site and various restriction cloning sites. The expression of the CBD-*Gaussia* luciferase is under the joint control of the lac operator and T7 promoter, which is inducible by IPTG.

**20      3) CBD-*Pleuromamma* luciferase in pET-34 (6485 bp)**

A CBD-*Pleuromamma* luciferase CDS was cloned into the pET-34 (Novagen), which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Kan), a phage replication origin (f1 origin), lacI sequence, T7 promoter, lac operator, rbs sequence, LIC site, 3' LIC overlap sequence, His tag CDS, His tag sequence, T7 terminator, thrombin cleavage site, S tag CDS sequence, EK cleavage site and various restriction cloning sites. Expression of the CBD-*Pleuromamma* luciferase is under the joint control of lac operator and T7 promoter, which is inducible by IPTG.

**25      4) CBD-*Ptilosarcus* GFP in pET-34 (6605 bp)**

A CBD-*Ptilosarcus* GFP CDS was cloned into the pET-34 (Novagen) which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Kan), a phage replication origin (f1 origin), lacI sequence, T7 promoter, lac operator, rbs sequence, LIC site, 3' LIC overlap sequence, His tag

-165-

CDS, His tag sequence, T7 terminator, thrombin cleavage site, S tag CDS sequence, EK cleavage site and various restriction cloning sites. Expression of the CBD-*Ptilosarcus* GFP is under the joint control of lac operator and T7 promoter, which is inducible by IPTG.

5           **5) CBD-*Renilla mulleri* GFP in pET-34 (6605 bp)**

A CBD-*Renilla mulleri* GFP CDS was cloned into the pET-34 (Novagen) which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Kan), a phage replication origin (f1 origin), lacI sequence, T7 promoter, lac operator, rbs sequence, LIC site, 3' LIC overlap sequence, His tag  
10 CDS, His tag sequence, T7 terminator, thrombin cleavage site, S tag CDS sequence, EK cleavage site and various restriction cloning sites. The expression of the CBD-*Renilla mulleri* GFP is under the joint control of the lac operator and T7 promoter, which is inducible by IPTG.

**F. Plasmid for expressing *Renilla mulleri* luciferase-GFP fusion protein**

15 As depicted in Figure 7, *Renilla mulleri* GFP was inserted into the ligation independent cloning site of the pET-34 vector (Novagen). The cellulose binding domain (CBD) that is normally present in pET-34 was deleted using inverse PCR. To facilitate optimization for analytical uses of the resulting fusion protein (such as for BRET), additional restriction sites (not shown) have been introduced into  
20 the linker region, permitting insertion of desired linking and target proteins or moieties. Using two of these sites, the *Renilla mulleri* luciferase CDS was inserted in the standard position of the CBD. In this plasmid, or in similar constructs that retain the CBD, near-native and native GFP can be cleaved from the fusion protein by treatment with thrombin or enterokinase (EK), respectively.  
25 The RNase S-peptide tag (S tag CDS) facilitates immunoaffinity purification of the GFP or the fusion protein, and allows quantitation of these proteins in crude extracts using a commercial RNase assay (Novagen). If the luciferase is separately fused to the S-tag in a second separate plasmid, association or co-expression of the S-protein/luciferase and S-protein/GFP via the RNase domain  
30 creates a test system for intermolecular BRET.

**G. Functional expression of *Renilla mulleri* GFP in mammalian cells**

-166-

HeLa cells were transfected with the plasmid pcDNA3.1(+) containing *Renilla mulleri* GFP under the control of a CMV promoter (construct No. B(1) as described above). HeLa cells were burst transfected with 1.5 micrograms of pcDNA3.1 per plate using the LipofectAMINE plus kit (GIBCO).

- 5        When HeLa cells were burst transfected with 30 micrograms of pcDNA-*Renilla mulleri* GFP DNA and grown at 37°C for 8 hours, a sub-population of cells were observed to be highly fluorescent. The fluorescence was localized to pairs of cells that were undergoing, or apparently had recently undergone, a single round of cell-division. This result indicates that native *Renilla mulleri* GFP
- 10       can be expressed, folded and complexed properly with the chromophore, and retain its function to produce green fluorescence in mammalian cells. This is in contrast to the *Aequorea* GFP, which folds inefficiently into a functional form under the physiological conditions.

- 15       Filters (470/40 excitation filter, 495 excitation dichroic filter and 525/50 emission filter) used in visualization of *R. mulleri* GFP fluorescence in HeLa cells were the Endo GFP filter set sold by Chroma.

- 20       Since modifications will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claims.

-167-

## Summary of the Sequence Listing

1. SEQ ID NO. 1 *Renilla reniformis* Luciferase [U.S. Patent No. 5,418,155]
2. SEQ ID NO. 2 *Cypridina hilgendorffii* luciferase [EP 0 387 355]
3. SEQ ID NO. 3 Modified *Luciola cruciata* Luciferase [firefly; U.S. Patent  
5 No. 4,968,613]
4. SEQ ID NO. 4 *Vargula (Cypridina)* luciferase [Thompson et al. (1989)  
Proc. Natl. Acad. Sci. U.S.A. 86:6567-6571 and from JP 3-30678  
Osaka]
5. SEQ ID NO. 5 Apoaequorin-encoding gene [U S. Patent No. 5,093,240,  
10 PAQ440]
6. SEQ ID NO. 6 Encoded *Aequorin* AEQ1 [Prasher et al. (1987) "Sequence  
Comparisons of CDNAS Encoding for Aequorin Isotypes," Biochemistry  
26:1326-1332]
7. SEQ ID NO. 7 Encoded *Aequorin* AEQ2 [Prasher et al. (1987)]
- 15 8. SEQ ID NO. 8 Encoded *Aequorin* AEQ3 [Prasher et al. (1987)]
9. SEQ ID NO. 9 *Aequorin* photoprotein [Charbonneau et al. (1985)  
"Amino Acid Sequence of the Calcium-Dependent Photoprotein  
Aequorin," Biochemistry 24:6762-6771]
10. SEQ ID NO. 10 *Aequorin* mutant with increased bioluminescence activity  
20 [U.S. Patent No. 5,360,728; Asp 124 changed to Ser]
11. SEQ ID NO. 11 *Aequorin* mutant with increased bioluminescence activity  
[U.S. Patent No. 5,360,728; Glu 135 changed to Ser]
12. SEQ ID NO. 12 *Aequorin* mutant with increased bioluminescence  
activity [U.S. Patent No. 5,360,728 Gly 129 changed to Ala]
- 25 13. SEQ ID NO. 13 Encoded apoaequorin [sold by Sealite, Sciences, Bogart,  
GA as AQUALITE®, when reconstituted to form aequorin]
14. SEQ ID NO. 14 *Vibrio fischeri* Flavin reductase [U.S. Patent No.  
5,484,723]
15. SEQ ID NO. 15 nucleic acid encoding *Renilla mulleri* green fluorescent  
30 protein (GFP)
16. SEQ ID NO. 16 Encoded *Renilla mulleri* green fluorescent protein (GFP)
17. SEQ ID NO. 17 nucleic acid encoding *Renilla mulleri* luciferase

-168-

18. SEQ ID NO. 18 Encoded *Renilla mulleri* luciferase
19. SEQ ID NO. 19 nucleic acid encoding *Gausssia* luciferase
20. SEQ ID NO. 20 Encoded *Gausssia* luciferase
21. SEQ ID NO. 21 nucleic acid encoding a *Gaussia* luciferase fusion protein
- 5 22. SEQ ID NO. 22 Encoded *Gaussia* luciferase fusion protein
23. SEQ ID NO. 23 *Renilla reniformis* GFP peptide corresponding to amino acid sequences near the amino-terminus of *R. mulleri* GFP
24. SEQ ID NO. 24 *Renilla reniformis* GFP peptide corresponding to amino acid sequences near the amino-terminus of *R. mulleri* GFP
- 10 25. SEQ ID NO. 25 *Renilla reniformis* GFP peptide corresponding to amino acid sequences near the middle of *R. mulleri* GFP
26. SEQ ID NO. 26 *Renilla reniformis* GFP peptide corresponding to amino acid sequences near the middle of *R. mulleri* GFP
27. SEQ ID NO. 27 *Renilla reniformis* GFP peptide corresponding to amino acid sequences near the carboxy-terminus of *R. mulleri* GFP
- 15 28. SEQ ID NO. 28 Pleuromamma luciferase\insert 861 bp
29. SEQ ID No. 29 encoded Pleuromamma luciferase 198 aa
30. SEQ ID NO:30 Ptilosarcus GFP\insertA\1104 bp
31. SEQ ID NO:31 Ptilosarcus GFP\insertB\1279 bp
- 20 32. SEQ ID NO:32 Ptilosarcus GFP 238 aa

-169-

## WHAT IS CLAIMED IS:

1. An isolated nucleic acid fragment, comprising a sequence of nucleotides encoding a *Renilla mulleri* luciferase, a *Gaussia* luciferase or a *Pleuromamma* luciferase.
- 5 2. The isolated nucleic acid fragment of claim 1, wherein the *Gaussia* is a member of the species of *princeps*.
3. The isolated nucleic acid fragment of claim 1, wherein the nucleic acid is DNA.
4. The isolated nucleic acid fragment of claim 1, wherein the nucleic  
10 acid is RNA.
5. An isolated nucleic acid fragment of claim 1, comprising the sequence of nucleotides selected from the group consisting of  
a sequence of nucleotides set forth in SEQ ID No. 17, SEQ ID No. 19, or SEQ ID No. 28;  
15 a sequence of nucleotides encoding the amino acid sequence set forth in SEQ ID No. 18, SEQ ID No. 20 or SEQ ID No. 29; and  
a sequence of nucleotides that hybridizes under high stringency to the sequence of nucleotides set forth in SEQ ID No. 17, SEQ ID No. 19 or SEQ ID No. 28.
- 20 6. A nucleic acid probe or primer, comprising at least 14 contiguous nucleotides selected from the sequence of nucleotides set forth in the coding portion of SEQ ID Nos. 17, 19 or 28.
7. The nucleic acid probe or primer of claim 6, comprising at least 16 contiguous nucleotides selected from the sequence of nucleotides set forth  
25 in the coding portion of SEQ ID Nos. 17, 19 or 28.
8. The nucleic acid probe or primer of claim 6, comprising at least 30 contiguous nucleotides selected from the sequence of nucleotides set forth in the coding portion of SEQ ID Nos. 17, 19 or 28.
9. A plasmid, comprising the nucleic acid fragment of claim 1.
- 30 10. The plasmid of claim 9 that is an expression vector.
11. The plasmid of claim 10, comprising a sequence of nucleotides encoding:

-170-

a promoter region;

a *Gaussia*, *Pleuromamma* or *Renilla mulleri* luciferase; and

a selectable marker;

wherein the sequence of nucleotides encoding the luciferase is operatively

5 linked to the promoter, whereby the luciferase is expressed.

12. The plasmid of claim 10, further comprising a sequence of nucleotides encoding a green fluorescent protein (GFP).

13. A recombinant host cell, comprising the plasmid of claim 10.

14. The cell of claim 13, wherein the cell is selected from the group  
10 consisting of a bacterial cell, a yeast cell, a fungal cell, a plant cell, an insect cell and an animal cell.

15. A method for producing a *Gaussia*, *Renilla mulleri* or *Pleuromamma* luciferase protein, comprising growing the recombinant host cell of claim 13, wherein the luciferase protein is expressed by the cell, and recovering the expressed luciferase protein.

16. An isolated substantially purified *Gaussia*, *Renilla mulleri* or *Pleuromamma* luciferase protein.

17. The isolated protein of claim 16, wherein the protein has the sequence of amino acids set forth in SEQ ID Nos. 18, 20 or 29.

20 18. A combination, comprising:

an article of manufacture; and

a bioluminescence generating system, whereby the combination is a novelty item, wherein the bioluminescence generating system comprises a luciferase encoded by the nucleic acid of claim 1.

25 19. The combination of claim 18, further comprising a luciferin.

20. The combination of claim 18, further comprising a green fluorescent protein (GFP).

21. The combination of claim 20, wherein the GFP is a *Renilla* GFP or a *Ptilocarpus* GFP.

30 22. The combination of claim 18, wherein the article of manufacture is selected from among toys, cosmetics, fountains, personal care items, fairy



-171-

dust, beverages, soft drinks, foods, textile products, bubbles, balloons, personal items, dentifrices, soaps, body paints, bubble bath, ink and paper products.

23. The combination of claim 22 that is a toy gun.
24. The combination of claim 22 that is a food.
- 5 25. The combination of claim 22 that is a beverage.
26. The combination of claim 22 that is a cosmetic.
27. The combination of claim 18, wherein the article of manufacture is selected from among squirt guns, pellet guns, finger paints, foot bags, greeting cards, slimy play material, clothing, bubble making toys, bath powders, 10 cosmetics, body lotions, gels, body powders, body creams, toothpastes, mouthwashes, soaps, body paints, bubble bath, inks, wrapping paper, gelatins, icings, frostings, greeting cards, beer, wine, champagne, soft drinks, ice cubes, ice, dry ice and fountains.
28. The combination of claim 27 that is a toy gun.
- 15 29. The combination of claim 27 that is a food.
30. The combination of claim 27 that is a beverage.
31. The combination of claim 27 that is a cosmetic.
32. An isolated nucleic acid fragment, comprising a sequence of nucleotides encoding a *Renilla* green fluorescent protein (GFP) or a *Ptilocarpus* 20 green fluorescent protein.
33. The isolated nucleic acid fragment of claim 32, wherein the *Renilla* species is selected from the group consisting of *Renilla reniformis*, *Renilla kollokeri* and *Renilla mulleri*.
34. The isolated nucleic acid fragment of claim 32, wherein the 25 nucleic acid is DNA.
35. The isolated nucleic acid fragment of claim 32, wherein the nucleic acid is RNA.
36. An isolated nucleic acid fragment encoding a green fluorescent protein (GFP), comprising the sequence of nucleotides selected from the group 30 consisting of  
a sequence of nucleotides set forth in SEQ ID No. 15, SEQ ID No. 30 or SEQ ID No. 31;

-172-

a sequence of nucleotides encoding the amino acid sequence set forth in SEQ ID No. 16 or SEQ ID No. 32; and

a sequence of nucleotides that hybridizes under high stringency to the sequence of nucleotides set forth in SEQ ID No. 15, SEQ ID No. 30 or SEQ

5 ID No. 31.

37. A nucleic acid probe or primer, comprising at least 14 contiguous nucleotides selected from the sequence of nucleotides set forth in SEQ ID No. 15, SEQ ID No. 30 or SEQ ID No. 31.

38. The probe or primer of claim 37, comprising at least 16  
10 contiguous nucleotides selected from the sequence of nucleotides set forth in SEQ ID No. 15, SEQ ID No. 30 or SEQ ID No. 31.

39. The probe or primer of claim 37, comprising at least 30 contiguous nucleotides selected from the sequence of nucleotides set forth in SEQ ID No. 15, SEQ ID No. 30 or SEQ ID No. 31.

15 40. A plasmid, comprising the sequence of nucleotides of claim 32.

41. An expression vector, comprising:

the plasmid of claim 40;

a promoter element;

a multiple cloning site for the introduction of nucleic acid; and

20 a selectable marker;

wherein the nucleic acid encoding the multiple cloning site is positioned between nucleic acids encoding the promoter element and the green fluorescent protein and wherein the nucleic acid encoding the green fluorescent protein is operatively linked to the promoter element.

25 42. The plasmid of claim 40, further comprising a sequence of nucleotides encoding:

a promoter element;

a selectable marker;

wherein, the sequence of nucleotides encoding the green fluorescent protein is  
30 operatively linked to the promoter element, whereby the green fluorescent protein is expressed.

-173-

43. The plasmid of claim 42, further comprising a sequence of nucleotides encoding a luciferase.
44. A recombinant host cell, comprising the plasmid of claim 40.
45. The cell of claim 44, wherein the cell is selected from the group  
5 consisting of a bacterial cell, a yeast cell, a fungal cell, a plant cell, an insect cell and an animal cell.
46. An isolated substantially purified *Renilla mulleri* green fluorescent protein (GFP) or a *Ptilosarcus* GFP.
47. A composition, comprising the green fluorescent protein of claim  
10 46.
48. The composition of claim 47, further comprising at least one component of a bioluminescence generating system.
49. The composition of claim 48, wherein the bioluminescence generating system is selected from those isolated from: an insect system, a  
15 coelenterate system, a ctenophore system, a bacterial system, a mollusk system, a crustacea system, a fish system, an annelid system, and an earthworm system.
50. The composition of claim 48, wherein the bioluminescence generating system is selected from those isolated from: fireflies, *Mnemiopsis*,  
20 *Beroe ovata*, *Aequorea*, *Obelia*, *Vargula*, *Pelagia*, *Renilla*, *Pholas*, *Aristostomias*, *Pachystomias*, *Porichthys*, *Cypridina*, *Aristostomias*, such *Pachystomias*, *Malacosteus*, *Gonadostomias*, *Gaussia*, *Watensia*, *Halisturia*, Vampire squid, *Glyphus*, Myctophids, *Vinciguerria*, *Howella*, *Florenciella*, *Chaudiodus*, *Melanocostus*, Sea Pens, *Chroteuthis*, *Eucleoteuthis*, *Onychoteuthis*,  
25 *Watasenia*, cuttlefish, *Sepiolina*, *Oplophorus*, *Acanthophyrea*, *Sergestes*, *Gnathophausia*, *Argyropelecus*, *Yarella*, *Diaphus*, *Gonadostomias* and *Neoscopelus*.
51. The composition of claim 50, wherein the bioluminescence generating system is selected from those isolated from *Aequorea*, *Obelia*,  
30 *Vargula* and *Renilla*.
52. A combination, comprising:  
an article of manufacture; and

-174-

a *Renilla* or *Ptilosarcus* green fluorescent protein (GFP).

53. The combination of claim 52, further comprising  
at least one component of a bioluminescence generating system,  
whereby the combination is a novelty item.
- 5 54. The combination of claim 53, wherein the combination comprises  
a luciferase.
55. The combination of claim 53, wherein the combination comprises  
a luciferin.
56. The combination of claim 53, wherein the combination comprises  
10 a luciferin and a luciferase.
57. The combination of claim 52, wherein the article of manufacture  
is selected from among toys, fountains, personal care items, cosmetics, fairy  
dust, beverages, soft drinks, foods, textile products, bubbles, balloons, personal  
items, dentifrices, soaps, body paints, bubble bath, ink and paper products.
- 15 58. The combination of claim 57 that is a toy gun.
59. The combination of claim 57 that is a food.
60. The combination of claim 57 that is a cosmetic.
61. The combination of claim 57 that is a beverage.
62. The combination of claim 52, wherein the article of manufacture  
20 is selected from among squirt guns, pellet guns, finger paints, foot bags, slimy  
play material, clothing, bubble making toys, bath powders, body lotions, gels,  
body powders, body creams, toothpastes, mouthwashes, soaps, body paints,  
bubble bath, inks, wrapping paper, gelatins, icings, frostings, beer, wine,  
champagne, soft drinks, ice cubes, ice, dry ice and fountains.
- 25 63. The combination of claim 62 that is a toy gun.
64. The combination of claim 62 that is a food.
65. The combination of claim 62 that is a cosmetic.
66. The combination of claim 62 that is a beverage.
67. An antibody that immuno-specifically binds a *Renilla mulleri*  
30 luciferase, a *Gaussia* luciferase or a *Pleuromamma* luciferase, or a fragment or  
derivative of said antibody containing the binding domain thereof.
68. The antibody of claim 67 which is a monoclonal antibody.

-175-

69. An antibody that immuno-specifically binds a *Renilla* green fluorescent protein (GFP) or a *Ptilocarpus* GFP, or a fragment or derivative of said antibody containing the binding domain thereof.

70. The antibody of claim 69, wherein the *Renilla* GFP is a *Renilla*  
5 *reniformis*, *Renilla kollokeri* or *Renilla mulleri* GFP.

71. The antibody of claim 69 which is a monoclonal antibody.

72. A nucleic acid construct, comprising a nucleotide sequence encoding a luciferase and a nucleotide sequence encoding a green fluorescent protein (GFP).

10 73. The nucleic acid construct of claim 72, wherein the luciferase is a *Renilla mulleri* luciferase, a *Gaussia* luciferase or a *Pleuromamma* luciferase.

74. The nucleic acid construct of claim 73, wherein the *Gaussia* luciferase is a *Gaussia princeps* luciferase.

75. The nucleic acid construct of claim 72, wherein the luciferase is  
15 encoded by:

a sequence of nucleotides set forth in SEQ ID No. 17, SEQ ID No. 19, or SEQ ID No. 28;

a sequence of nucleotides encoding the amino acid sequence set forth in SEQ ID No. 18, SEQ ID No. 20 or SEQ ID No. 29; and

20 a sequence of nucleotides that hybridizes under high stringency to the sequence of nucleotides set forth in SEQ ID No. 17, SEQ ID No. 19 or SEQ ID No. 28.

76. The nucleic acid construct of claim 72, wherein the GFP is a *Renilla* green fluorescent protein (GFP) or a *Ptilocarpus* GFP.

25 77. The nucleic acid construct of claim 76, wherein the *Renilla* GFP is a *Renilla reniformis*, *Renilla kollokeri* or *Renilla mulleri* GFP.

78. The nucleic acid construct of claim 72, wherein the GFP is encoded by:

a sequence of nucleotides set forth in SEQ ID No. 15, SEQ ID No.  
30 30 or SEQ ID No. 31;

a sequence of nucleotides encoding the amino acid sequence set forth in SEQ ID No. 16 or SEQ ID No. 32; and

-176-

a sequence of nucleotides that hybridizes under high stringency to the sequence of nucleotides set forth in SEQ ID No. 15, SEQ ID No. 30 or SEQ ID No. 31.

79. The nucleic acid construct of claim 72, wherein the luciferase and  
5 GFP are from *Renilla*.

80. The nucleic acid construct of claim 79, wherein the *Renilla* luciferase and GFP are from *Renilla mulleri*.

81. The nucleic acid construct of claim 72, wherein the nucleotide sequence encoding the luciferase and GFP are linked contiguously.

10 82. The nucleic acid construct of claim 72 which is DNA.

83. The nucleic acid construct of claim 72 which is RNA.

84. A plasmid, comprising the nucleic acid construct of claim 72.

85. The plasmid of claim 84, further comprising a sequence of nucleotides encoding:

15 a promoter element;

a selectable marker;

wherein, the sequence of nucleotides encoding the luciferase and GFP is operatively linked to the promoter element, whereby a fusion protein of the luciferase and GFP is expressed.

20 86. A recombinant host cell, comprising the plasmid of claim 84.

87. The cell of claim 86, wherein the cell is selected from the group consisting of a bacterial cell, a yeast cell, a fungal cell, a plant cell, an insect cell and an animal cell.

25 88. An isolated substantially purified luciferase and GFP fusion protein.

89. The fusion protein of claim 88, wherein the luciferase and GFP are from *Renilla*.

90. The fusion protein of claim 89, wherein the *Renilla* luciferase and GFP are from *Renilla mulleri*.

30 91. A composition, comprising the fusion protein of claim 88.

92. The composition of claim 91, further comprising at least one component of a bioluminescence generating system.

-177-

93. The composition of claim 92, wherein the component of the bioluminescence generating system is luciferin.

94. The nucleic acid construct of claim 72, wherein the nucleotide sequence encoding the luciferase and GFP are not contiguous.

5 95. The nucleic acid construct of claim 94, comprising a sequence of nucleotides that encodes a ligand binding domain of a target protein.

96. A nucleic acid probe or primer, comprising at least 14 nucleotides, preferably 16 to 30 nucleotides, that are based on amino acids 51 to 68, 82 to 98 and 198 to 208 set forth in SEQ ID No. 16, amino acid sequence set forth in  
10 SEQ ID No. 24, amino acids 9-20 set forth in SEQ ID No. 25 or amino acids 39-53 set forth in SEQ ID No. 27.

97. The probe or primer of claim 96, comprising a sequence of nucleic acids in SEQ ID No.15 that encodes the sequence of amino acids.

98. A method of isolating nucleic acid that encodes a *Renilla* green  
15 fluorescent protein (GFP), comprising:  
screening a *Renilla* nucleic acid library with a probe or plurality of probes of claim 96; and

identifying and isolating nucleic acid that encodes a GFP.

99. A method of isolating nucleic acid that encodes a *Renilla* green  
20 fluorescent protein (GFP), comprising:

amplifying nucleic acid in a *Renilla* nucleic acid library with a primer or plurality of primers of claim 96; and

isolating the amplified nucleic acids, whereby nucleic acid encoding a GFP is identified and isolated.

1/11

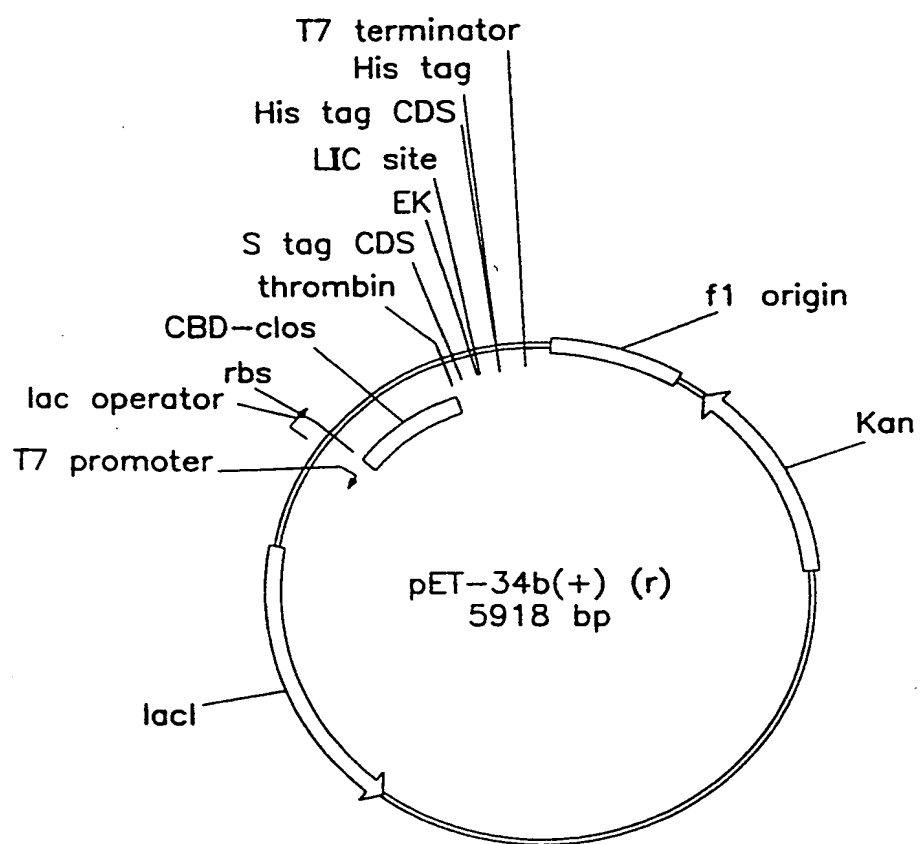


FIG. 1



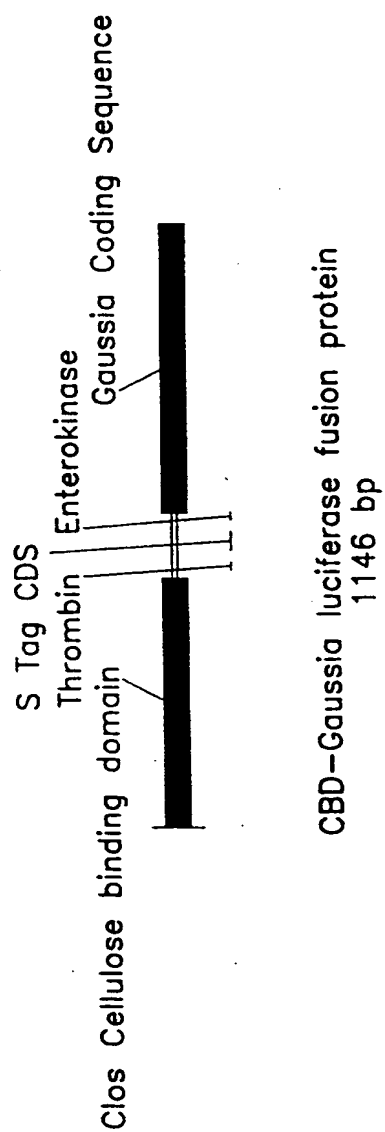


FIG. 2

FIG. 3

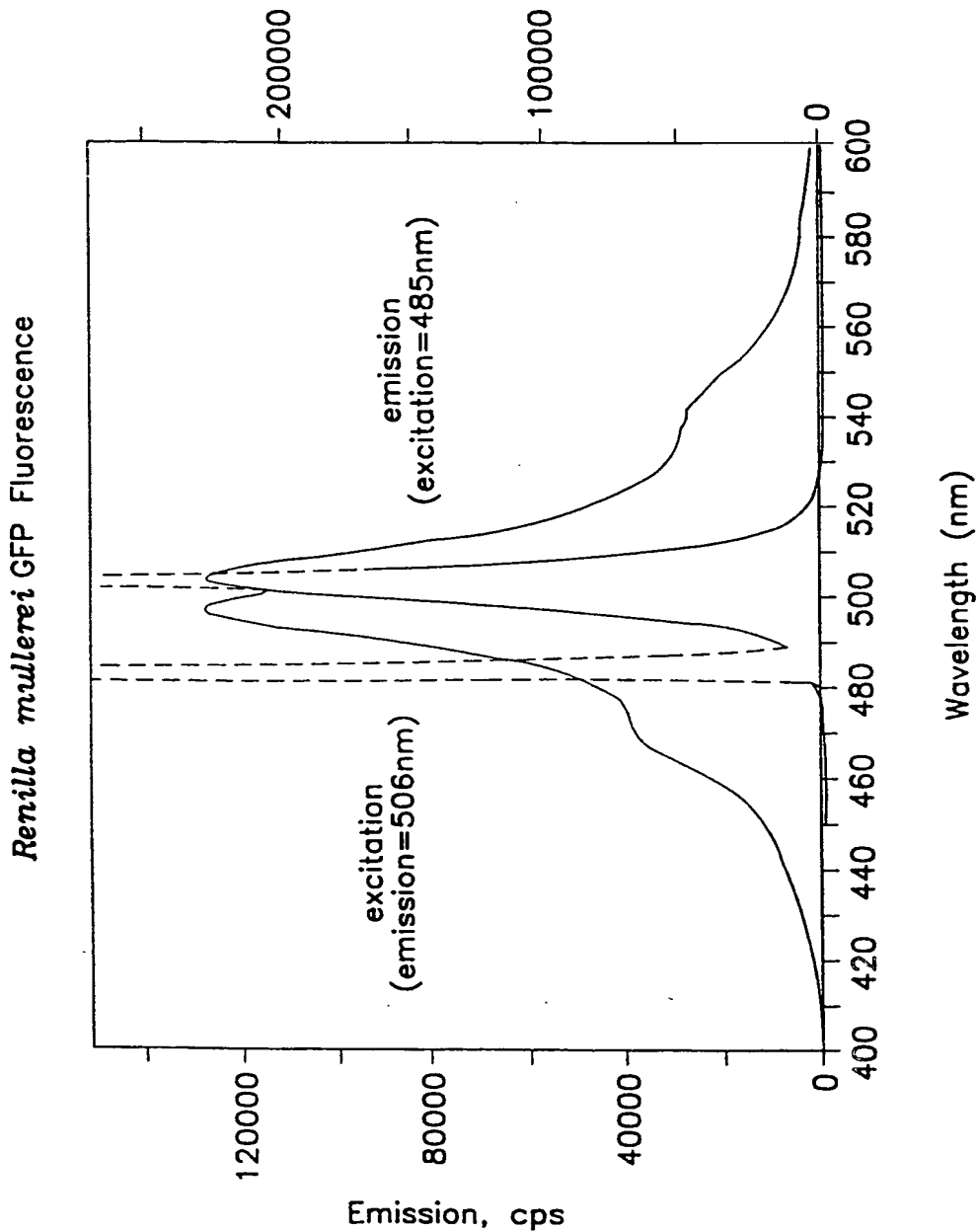


FIG. 4

5/11

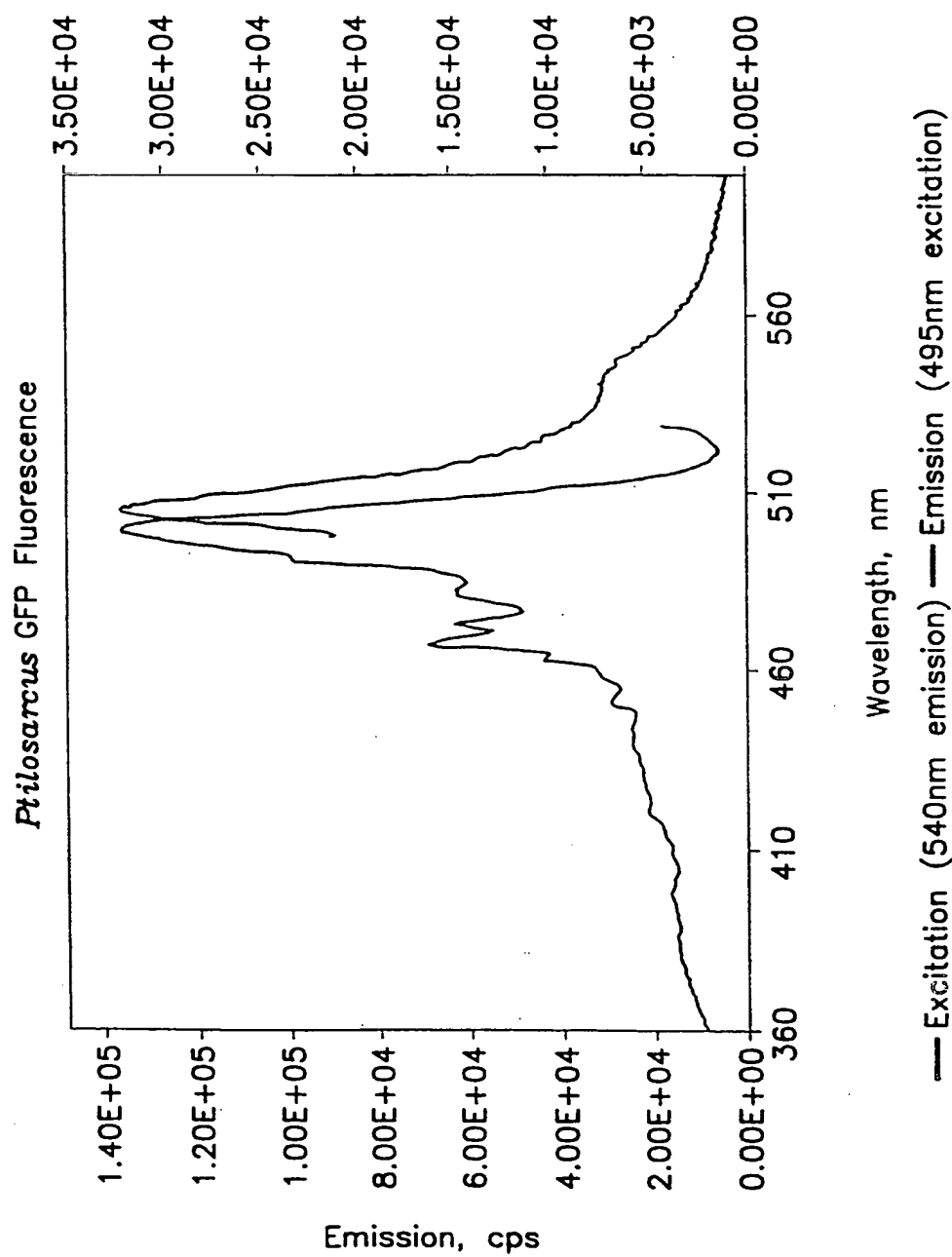


FIG. 5

6/11

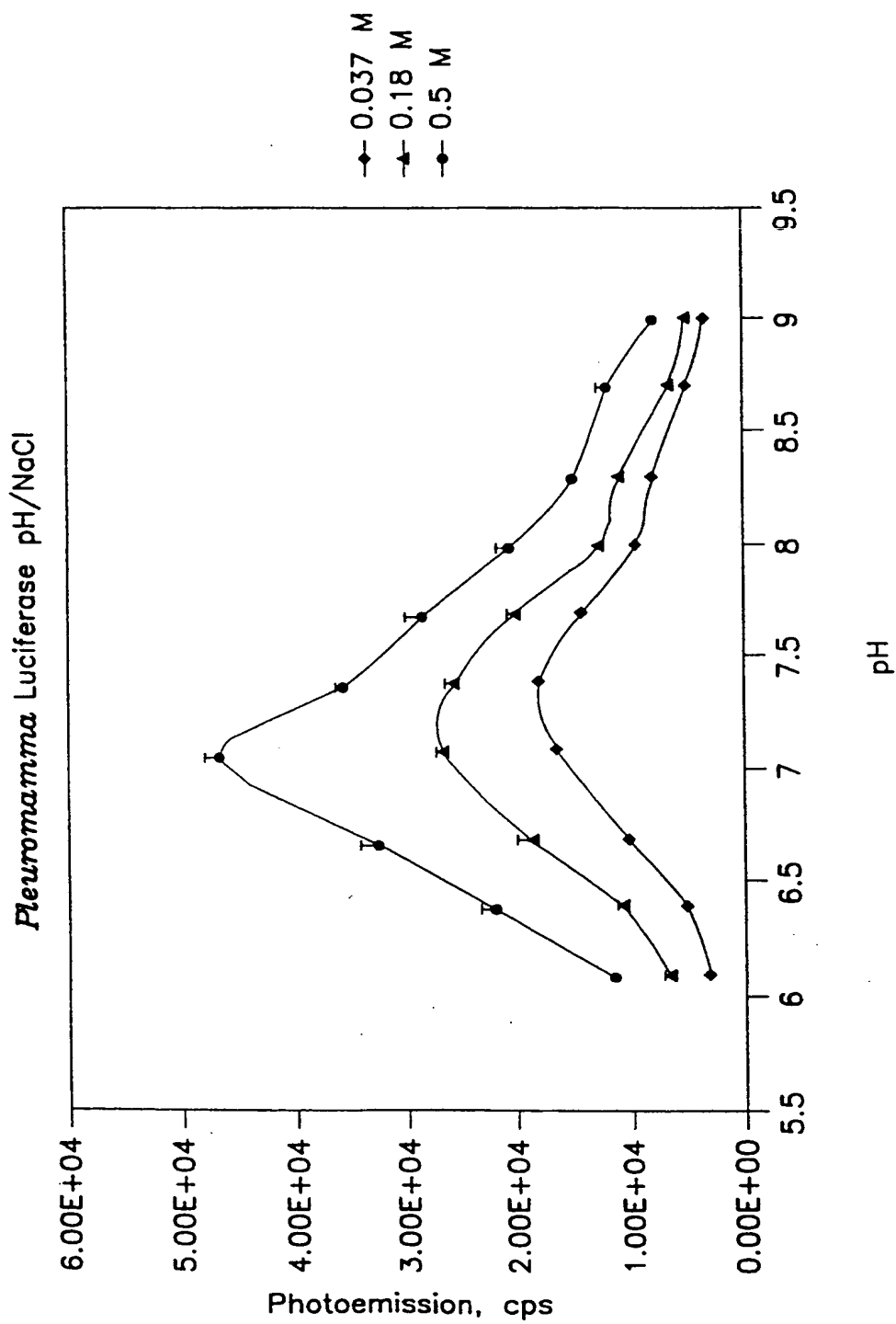
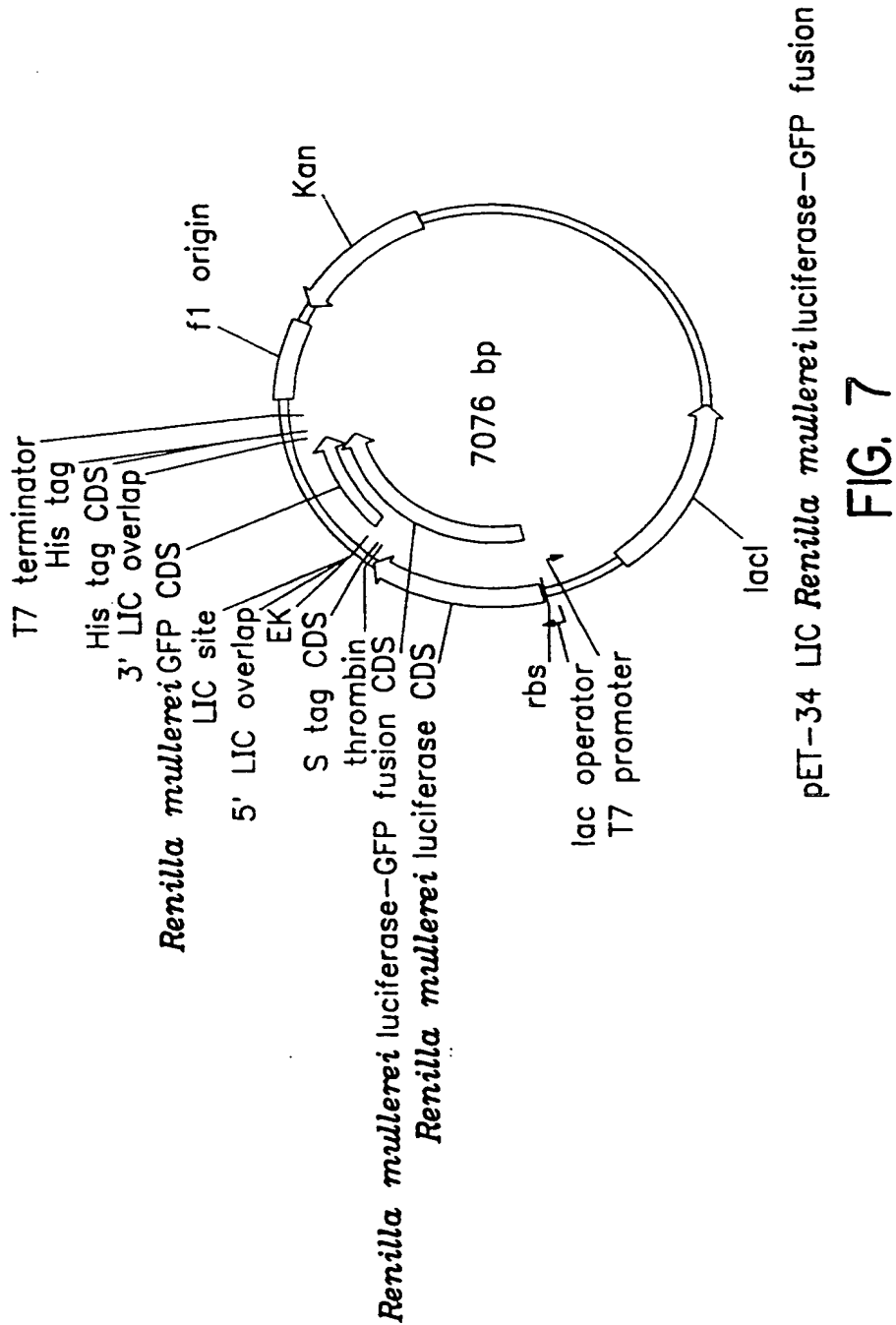


FIG. 6



8/11

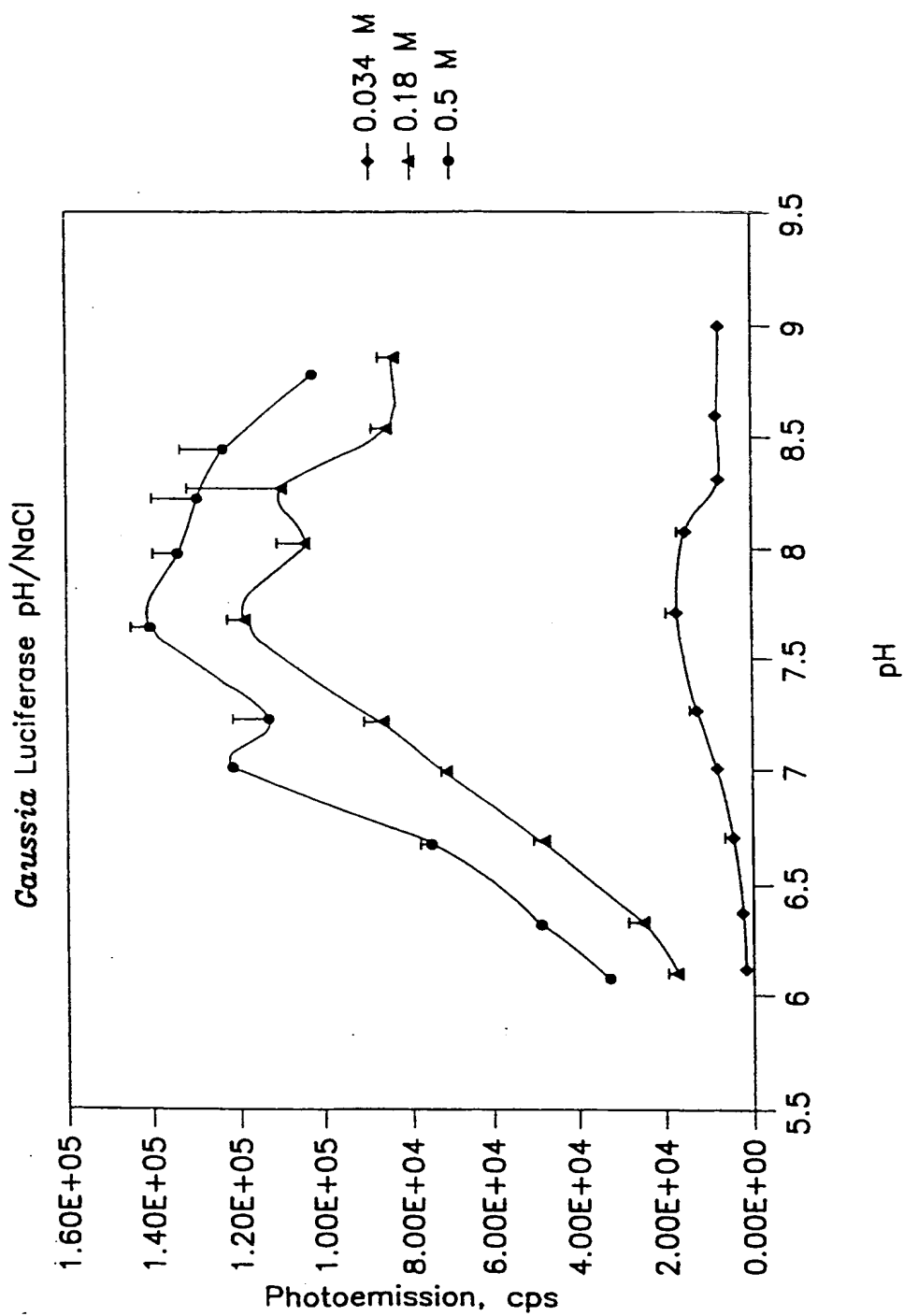


FIG. 8

9/11

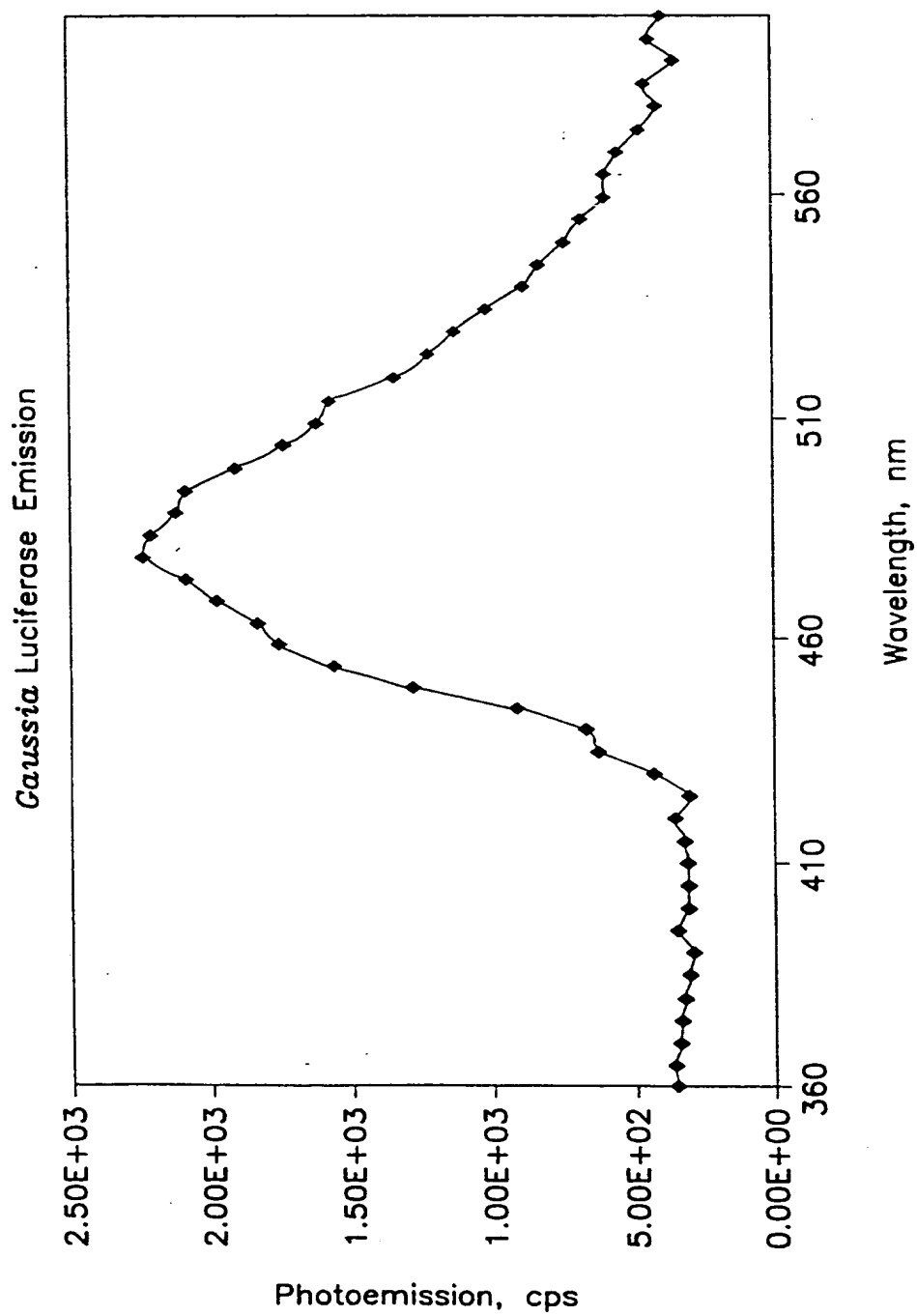


FIG. 9



10/11

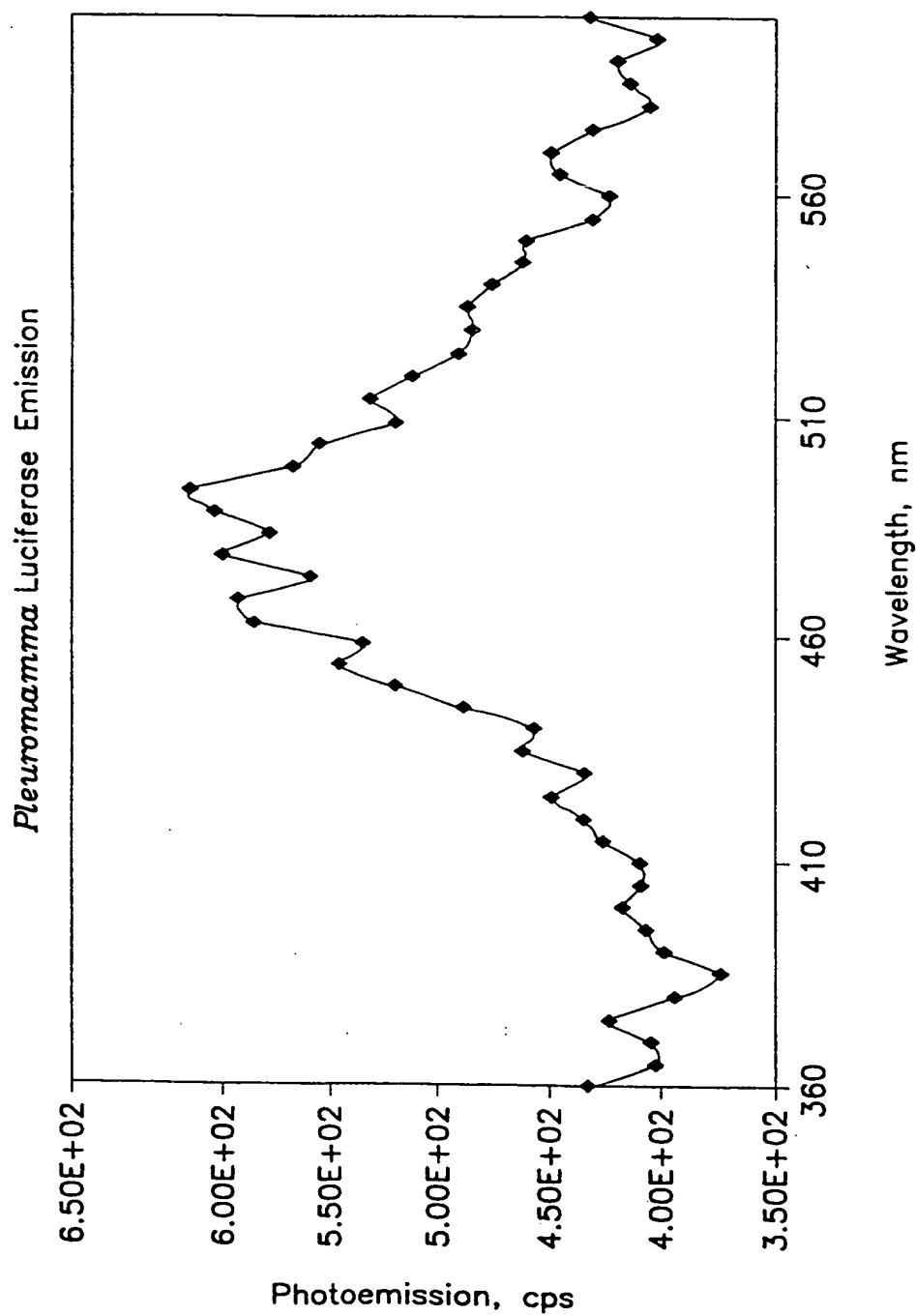


FIG. 10

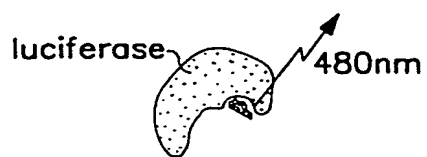


FIG. 1 IA

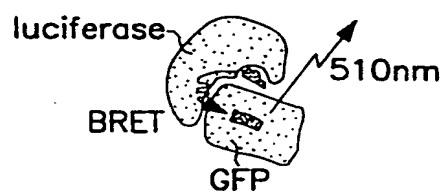


FIG. 1 IC

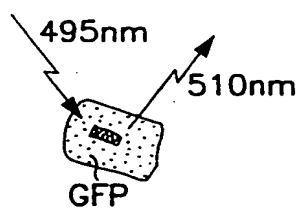


FIG. 1 IB

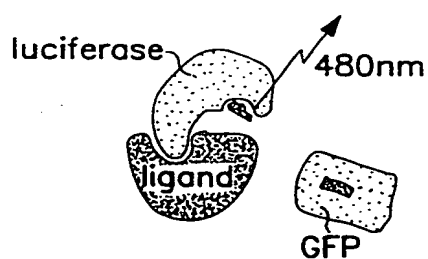


FIG. 1 ID

-1/43-

## SEQUENCE LISTING

## (1) GENERAL INFORMATION

- (i) APPLICANT:
  - (A) NAME: PROLUME, LTD.
  - (B) STREET: 1085 William Pitt Way
  - (D) STATE: Pennsylvania
  - (D) COUNTRY: USA
  - (E) POSTAL CODE (ZIP): 15236
- (i) INVENTOR/APPLICANT:
  - (A) NAME: Bryan, Bruce
  - (B) STREET: 716 Arden Drive
  - (C) CITY: Beverly Hills
  - (D) STATE: California
  - (D) COUNTRY: USA
  - (E) POSTAL CODE (ZIP): 90210
- (i) INVENTOR/APPLICANT:
  - (A) NAME: Szent-Gyorgyi, Christopher
  - (B) STREET: 719 Duncan Avenue
  - (C) CITY: Pittsburgh
  - (D) STATE: Pennsylvania
  - (D) COUNTRY: USA
  - (E) POSTAL CODE (ZIP): 15237
- (ii) TITLE OF INVENTION: LUCIFERASES, FLUORESCENT PROTEINS,  
NUCLEIC ACIDS ENCODING THE LUCIFERASES  
AND FLUORESCENT PROTEINS AND THE USE  
THEREOF IN DIAGNOSTICS, HIGH THROUGHPUT  
SCREENING AND NOVELTY ITEMS
- (iii) NUMBER OF SEQUENCES: 32
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Heller Ehrman White & McAuliffe
  - (B) STREET: 4250 Executive Square, 7th Floor
  - (C) CITY: La Jolla
  - (D) STATE: CA
  - (E) COUNTRY: USA
  - (F) ZIP: 92037
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE:
  - (B) COMPUTER: IBM Compatible
  - (C) OPERATING SYSTEM: DOS
  - (D) SOFTWARE: FastSEQ Version 1.5 and ASCII
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE: 26-MAR-1999
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: 60/102,939
  - (B) FILING DATE: 01-OCT-1998
- (vii) PRIOR APPLICATION DATA:

-2/43-

- (A) APPLICATION NUMBER: 60/089,367  
 (B) FILING DATE: 15-JUNE-1998

- (vii) PRIOR APPLICATION DATA:  
 (A) APPLICATION NUMBER: 60/079,624  
 (B) FILING DATE: 27-MAR-1998

- (viii) ATTORNEY/AGENT INFORMATION:  
 (A) NAME: Seidman, Stephanie L  
 (B) REGISTRATION NUMBER: 33,779  
 (C) REFERENCE/DOCKET NUMBER: 24729-P121

- (ix) TELECOMMUNICATION INFORMATION:  
 (A) TELEPHONE: 619 450-8400  
 (B) TELEFAX: 619-450-8499  
 (C) TELEX:

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1196 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (vi) ORIGINAL SOURCE:

- (ix) FEATURE:

- (A) NAME/KEY: Coding Sequence  
 (B) LOCATION: 1...942  
 (D) OTHER INFORMATION: Renilla Reinformis Luciferase

- (x) PUBLICATION INFORMATION:

PATENT NO.: 5,418,155

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AGC TTA AAG ATG ACT TCG AAA GTT TAT GAT CCA GAA CAA AGG AAA CGG	48
Ser Leu Lys Met Thr Ser Lys Val Tyr Asp Pro Glu Gln Arg Lys Arg	
1 5 10 15	
ATG ATA ACT GGT CCG CAG TGG TGG GCC AGA TGT AAA CAA ATG AAT GTT	96
Met Ile Thr Gly Pro Gln Trp Trp Ala Arg Cys Lys Gln Met Asn Val	
20 25 30	
CTT GAT TCA TTT ATT AAT TAT TAT GAT TCA GAA AAA CAT GCA GAA AAT	144
Leu Asp Ser Phe Ile Asn Tyr Tyr Asp Ser Glu Lys His Ala Glu Asn	
35 40 45	
GCT GTT ATT TTT TTA CAT GGT AAC GCG GCC TCT TCT TAT TTA TGG CGA	192
Ala Val Ile Phe Leu His Gly Asn Ala Ala Ser Ser Tyr Leu Trp Arg	
50 55 60	
CAT GTT GTG CCA CAT ATT GAG CCA GTA GCG CGG TGT ATT ATA CCA GAT	240
His Val Val Pro His Ile Glu Pro Val Ala Arg Cys Ile Ile Pro Asp	
65 70 75 80	

-3/43-

CTT ATT GGT ATG GGC AAA TCA GGC AAA TCT GGT AAT GGT TCT TAT AGG Leu Ile Gly Met Gly Lys Ser Gly Lys Ser Gly Asn Gly Ser Tyr Arg 85 90 95	288
TTA CTT GAT CAT TAC AAA TAT CTT ACT GCA TGG TTG AAC TTC TTA ATT Leu Leu Asp His Tyr Lys Tyr Leu Thr Ala Trp Leu Asn Phe Leu Ile 100 105 110	336
TAC CAA AGA AGA TCA TTT TTT GTC GGC CAT GAT TGG GGT GCT TGT TTG Tyr Gln Arg Arg Ser Phe Phe Val Gly His Asp Trp Gly Ala Cys Leu 115 120 125	384
GCA TTT CAT TAT AGC TAT GAG CAT CAA GAT AAG ATC AAA GCA ATA GTT Ala Phe His Tyr Ser Tyr Glu His Gln Asp Lys Ile Lys Ala Ile Val 130 135 140	432
CAC GCT GAA AGT GTA GTA GAT GTG ATT GAA TCA TGG GAT GAA TGG CCT His Ala Glu Ser Val Val Asp Val Ile Glu Ser Trp Asp Glu Trp Pro 145 150 155 160	480
GAT ATT GAA GAA GAT ATT GCG TTG ATC AAA TCT GAA GAA GGA GAA AAA Asp Ile Glu Glu Asp Ile Ala Leu Ile Lys Ser Glu Glu Gly Glu Lys 165 170 175	528
ATG GTT TTG GAG AAT AAC TTC TTC GTG GAA ACC ATG TTG CCA TCA AAA Met Val Leu Glu Asn Asn Phe Phe Val Glu Thr Met Leu Pro Ser Lys 180 185 190	576
ATC ATG AGA AAG TTA GAA CCA GAA GAA TTT GCA GCA TAT CTT GAA CCA Ile Met Arg Lys Leu Glu Pro Glu Glu Phe Ala Ala Tyr Leu Glu Pro 195 200 205	624
TTC AAA GAG AAA GGT GAA GTT CGT CGT CCA ACA TTA TCA TGG CCT CGT Phe Lys Glu Lys Gly Glu Val Arg Arg Pro Thr Leu Ser Trp Pro Arg 210 215 220	672
GAA ATC CCG TTA GTA AAA GGT GGT AAA CCT GAC GTT GTA CAA ATT GTT Glu Ile Pro Leu Val Lys Gly Gly Lys Pro Asp Val Val Gln Ile Val 225 230 235 240	720
AGG AAT TAT AAT GCT TAT CTA CGT GCA AGT GAT GAT TTA CCA AAA ATG Arg Asn Tyr Asn Ala Tyr Leu Arg Ala Ser Asp Asp Leu Pro Lys Met 245 250 255	768
TTT ATT GAA TCG GAT CCA GGA TTC TTT TCC AAT GCT ATT GTT GAA GGC Phe Ile Glu Ser Asp Pro Gly Phe Phe Ser Asn Ala Ile Val Glu Gly 260 265 270	816
GCC AAG AAG TTT CCT AAT ACT GAA TTT GTC AAA GTA AAA GGT CTT CAT Ala Lys Lys Phe Pro Asn Thr Glu Phe Val Lys Val Lys Gly Leu His 275 280 285	864
TTT TCG CAA GAA GAT GCA CCT GAT GAA ATG GGA AAA TAT ATC AAA TCG Phe Ser Gln Glu Asp Ala Pro Asp Glu Met Gly Lys Tyr Ile Lys Ser 290 295 300	912
TTC GTT GAG CGA GTT CTC AAA AAT GAA CAA TAA TTACTTTGGT TTTTATTTA Phe Val Glu Arg Val Leu Lys Asn Glu Gln 305 310	965
CATTTTTCCC GGGTTTAATA ATATAAATGT CATTTTCAAC AATTTTATTT TAACTGAATA	1025

-4/43-

TTTCACAGGG AACATTCATA TATGTTGATT AATTTAGCTC GAACTTTACT CTGTCATATC 1085  
 ATTTTGGAAAT ATTACCTCTT TCAATGAAAC TTTATAAACA GTGGTTCAAT TAATTAATAT 1145  
 ATATTATAAT TACATTTGTT ATGTAATAAA CTCGGTTTTA TTATAAAAAA A 1196

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1822 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

- (A) NAME/KEY: Coding Sequence  
 (B) LOCATION: 1...1665  
 (D) OTHER INFORMATION: Cypridina hilgendorffii luciferase

## (x). PUBLICATION INFORMATION:

PATENT NO.: EP 0 387 355 TORAY

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATG AAG CTA ATA ATT CTG TCT ATT ATA TTG GCC TAC TGT GTC ACA GTC	48
Met Lys Leu Ile Ile Leu Ser Ile Ile Leu Ala Tyr Cys Val Thr Val	
1 5 10 15	
AAC TGC CAG GAT GCA TGT CCT GTA GAA GCT GAA GCA CCG TCA AGT ACA	96
Asn Cys Gln Asp Ala Cys Pro Val Glu Ala Glu Ala Pro Ser Ser Thr	
20 25 30	
CCA ACA GTC CCA ACA TCT TGT GAA GCT AAA GAA GGA GAA TGT ATC GAT	144
Pro Thr Val Pro Thr Ser Cys Glu Ala Lys Glu Gly Glu Cys Ile Asp	
35 40 45	
ACC AGA TGC GCA ACA TGT AAA CGA GAC ATA CTA TCA GAC GGA CTG TGT	192
Thr Arg Cys Ala Thr Cys Lys Arg Asp Ile Leu Ser Asp Gly Leu Cys	
50 55 60	
GAA AAT AAA CCA GGG AAG ACA TGC TGT AGA ATG TGC CAG TAT GTA ATT	240
Glu Asn Lys Pro Gly Lys Thr Cys Cys Arg Met Cys Gln Tyr Val Ile	
65 70 75 80	
GAA TCC AGA GTA GAA GCT GCT GGA TAT TTT AGA ACG TTT TAC GCC AAA	288
Glu Ser Arg Val Glu Ala Ala Gly Tyr Phe Arg Thr Phe Tyr Ala Lys	
85 90 95	
AGA TTT AAT TTT CAG GAA CCT GGT AAA TAT GTG CTG GCT CGA GGA ACC	336
Arg Phe Asn Phe Gln Glu Pro Gly Lys Tyr Val Leu Ala Arg Gly Thr	
100 105 110	
AAG GGT GGC GAC TGG TCT GTA ACC CTC ACC ATG GAG AAT CTA GAT GGA	384
Lys Gly Gly Asp Trp Ser Val Thr Leu Thr Met Glu Asn Leu Asp Gly	
115 120 125	
CAG AAG GGA GCT GTA CTG ACT AAG ACA ACA CTG GAG GTA GTA GGA GAC	432
Gln Lys Gly Ala Val Leu Thr Lys Thr Thr Leu Glu Val Val Gly Asp	
130 135 140	

-5/43-

GTA ATA GAC ATT ACT CAA GCT ACT GCA GAT CCT ATC ACA GTT AAC GGA Val Ile Asp Ile Thr Gln Ala Thr Ala Asp Pro Ile Thr Val Asn Gly 145 150 155 160	480
GGA GCT GAC CCA GTT ATC GCT AAC CCG TTC ACA ATT GGT GAG GTG ACC Gly Ala Asp Pro Val Ile Ala Asn Pro Phe Thr Ile Gly Glu Val Thr 165 170 175	528
ATT GCT GTT GTC GAA ATA CCC GGC TTC AAT ATT ACA GTC ATC GAA TTC Ile Ala Val Val Glu Ile Pro Gly Phe Asn Ile Thr Val Ile Glu Phe 180 185 190	576
TTT AAA CTA ATC GTG ATA GAT ATT CTG GGA GGA AGA TCT GTG AGA ATT Phe Lys Leu Ile Val Ile Asp Ile Leu Gly Gly Arg Ser Val Arg Ile 195 200 205	624
GCT CCA GAC ACA GCA AAC AAA GGA CTG ATA TCT GGT ATC TGT GGT AAT Ala Pro Asp Thr Ala Asn Lys Gly Leu Ile Ser Gly Ile Cys Gly Asn 210 215 220	672
CTG GAG ATG AAT GAC GCT GAT GAC TTT ACT ACA GAC GCA GAT CAG CTG Leu Glu Met Asn Asp Ala Asp Asp Phe Thr Thr Asp Ala Asp Gln Leu 225 230 235 240	720
GCG ATC CAA CCC AAC ATA AAC AAA GAG TTC GAC GGC TGC CCA TTC TAC Ala Ile Gln Pro Asn Ile Asn Lys Glu Phe Asp Gly Cys Pro Phe Tyr 245 250 255	768
GGG AAT CCT TCT GAT ATC GAA TAC TGC AAA GGT CTC ATG GAG CCA TAC Gly Asn Pro Ser Asp Ile Glu Tyr Cys Lys Gly Leu Met Glu Pro Tyr 260 265 270	816
AGA GCT GTA TGT CGT AAC AAT ATC AAC TTC TAC TAT TAC ACT CTG TCC Arg Ala Val Cys Arg Asn Asn Ile Asn Phe Tyr Tyr Tyr Thr Leu Ser 275 280 285	864
TGC GCC TTC GCT TAC TGT ATG GGA GGA GAA GAA AGA GCT AAA CAC GTC Cys Ala Phe Ala Tyr Cys Met Gly Gly Glu Glu Arg Ala Lys His Val 290 295 300	912
CTT TTC GAC TAT GTT GAG ACA TGC GCT GCA CCG GAA ACG AGA GGA ACG Leu Phe Asp Tyr Val Glu Thr Cys Ala Ala Pro Glu Thr Arg Gly Thr 305 310 315 320	960
TGT GTT TTA TCA GGA CAT ACT TTC TAT GAC ACA TTC GAC AAA GCC AGA Cys Val Leu Ser Gly His Thr Phe Tyr Asp Thr Phe Asp Lys Ala Arg 325 330 335	1008
TAT CAA TTC CAG GGC CCA TGC AAA GAG CTT CTG ATG GCC GCA GAC TGT Tyr Gln Phe Gln Gly Pro Cys Lys Glu Leu Leu Met Ala Ala Asp Cys 340 345 350	1056
TAC TGG AAC ACA TGG GAT GTA AAG GTT TCA CAT AGA GAT GTT GAG TCA Tyr Trp Asn Thr Trp Asp Val Lys Val Ser His Arg Asp Val Glu Ser 355 360 365	1104
TAC ACT GAG GTA GAG AAA GTA ACA ATC AGG AAA CAG TCA ACT GTA GTA Tyr Thr Glu Val Glu Lys Val Thr Ile Arg Lys Gln Ser Thr Val Val 370 375 380	1152
GAT TTG ATT GTG GAT GGC AAG CAG GTC AAG GTT GGA GGA GTG GAT GTA	1200

-6/43-

Asp	Leu	Ile	Val	Asp	Gly	Lys	Gln	Val	Lys	Val	Gly	Gly	Val	Asp	Val		
385					390					395					400		
TCT	ATC	CCG	TAC	AGT	TCT	GAG	AAC	ACA	TCC	ATA	TAC	TGG	CAG	GAT	GGA	1248	
Ser	Ile	Pro	Tyr	Ser	Ser	Glu	Asn	Thr	Ser	Ile	Tyr	Trp	Gln	Asp	Gly		
				405					410					415			
GAC	ATC	CTG	ACG	ACG	GCC	ATC	CTA	CCT	GAA	GCT	CTT	GTC	GTT	AAG	TTC	1296	
Asp	Ile	Leu	Thr	Thr	Ala	Ile	Leu	Pro	Glu	Ala	Leu	Val	Val	Lys	Phe		
				420				425					430				
AAC	TTT	AAG	CAG	CTC	CTT	GTA	GTT	CAT	ATC	AGA	GAT	CCA	TTC	GAT	GGA	1344	
Asn	Phe	Lys	Gln	Leu	Leu	Val	Val	His	Ile	Arg	Asp	Pro	Phe	Asp	Gly		
				435				440					445				
AAG	ACA	TGC	GGC	ATA	TGT	GGT	AAC	TAT	AAT	CAA	GAT	TCA	ACT	GAT	GAT	1392	
Lys	Thr	Cys	Gly	Ile	Cys	Gly	Asn	Tyr	Asn	Gln	Asp	Ser	Thr	Asp	Asp		
				450				455				460					
TTC	TTT	GAC	GCA	GAA	GGA	GCA	TGC	GCT	CTG	ACC	CCC	AAT	CCC	CCA	GGA	1440	
Phe	Phe	Asp	Ala	Glu	Gly	Ala	Cys	Ala	Leu	Thr	Pro	Asn	Pro	Pro	Gly		
					470					475					480		
TGT	ACA	GAG	GAG	CAG	AAA	CCA	GAA	GCT	GAG	CGA	CTC	TGC	AAT	AGT	CTA	1488	
Cys	Thr	Glu	Glu	Gln	Lys	Pro	Glu	Ala	Glu	Arg	Leu	Cys	Asn	Ser	Leu		
					485				490					495			
TTT	GAT	AGT	TCT	ATC	GAC	GAG	AAA	TGT	AAT	GTC	TGC	TAC	AAG	CCT	GAC	1536	
Phe	Asp	Ser	Ser	Ile	Asp	Glu	Lys	Cys	Asn	Val	Cys	Tyr	Lys	Pro	Asp		
				500				505						510			
CGT	ATT	GCA	CGA	TGT	ATG	TAC	GAG	TAT	TGC	CTG	AGG	GGA	CAG	CAA	GGA	1584	
Arg	Ile	Ala	Arg	Cys	Met	Tyr	Glu	Tyr	Cys	Leu	Arg	Gly	Gln	Gln	Gly		
				515				520					525				
TTC	TGT	GAC	CAT	GCT	TGG	GAG	TTC	AAA	AAA	GAA	TGC	TAC	ATA	AAG	CAT	1632	
Phe	Cys	Asp	His	Ala	Trp	Glu	Phe	Lys	Lys	Glu	Cys	Tyr	Ile	Lys	His		
				530				535					540				
GGA	GAC	ACT	CTA	GAA	GTA	CCA	CCT	GAA	TGC	CAA	TAA	ATGAACAAAG				1678	
Gly	Asp	Thr	Leu	Glu	Val	Pro	Pro	Glu	Cys	Gln							
					550					555							
ATACAGAAGC	TAAGACTACT	ACAGCAGAAG	ATAAAAGAGA	AGCTGTAGTT	CTTCAAAAAC	1738											
AGTATATTTT	GATGTACTCA	TTGTTTACTT	ACATAAAAAT	AAATTGTTAT	TATCATAACG	1798											
TAAAGAAAAA	AAAAAAAAAA	AAAAA				1822											

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1644 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
- (B) LOCATION: 1...1644



-7/43-

(D) OTHER INFORMATION: *Luciola Cruciata* Luciferase (Firefly)

(x) PUBLICATION INFORMATION:

PATENT NO.: 4,968,613

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATG GAA AAC ATG GAA AAC GAT GAA AAT ATT GTA GTT GGA CCT AAA CCG	48
Met Glu Asn Met Glu Asn Asp Glu Asn Ile Val Val Gly Pro Lys Pro	
1 5 10 15	
TTT TAC CCT ATC GAA GAG GGA TCT GCT GGA ACA CAA TTA CGC AAA TAC	96
Phe Tyr Pro Ile Glu Glu Gly Ser Ala Gly Thr Gln Leu Arg Lys Tyr	
20 25 30	
ATG GAG CGA TAT GCA AAA CTT GGC GCA ATT GCT TTT ACA AAT GCA GTT	144
Met Glu Arg Tyr Ala Lys Leu Gly Ala Ile Ala Phe Thr Asn Ala Val	
35 40 45	
ACT GGT GTT GAT TAT TCT TAC GCC GAA TAC TTG GAG AAA TCA TGT TGT	192
Thr Gly Val Asp Tyr Ser Tyr Ala Glu Tyr Leu Glu Lys Ser Cys Cys	
50 55 60	
CTA GGA AAA GCT TTG CAA AAT TAT GGT TTG GTT GTT GAT GGC AGA ATT	240
Leu Gly Lys Ala Leu Gln Asn Tyr Gly Leu Val Val Asp Gly Arg Ile	
65 70 75 80	
GCG TTA TGC AGT GAA AAC TGT GAA GAA TTT TTT ATT CCT GTA ATA GCC	288
Ala Leu Cys Ser Glu Asn Cys Glu Glu Phe Phe Ile Pro Val Ile Ala	
85 90 95	
GGA CTG TTT ATA GGT GTA GGT GTT GCA CCC ACT AAT GAG ATT TAC ACT	336
Gly Leu Phe Ile Gly Val Gly Val Ala Pro Thr Asn Glu Ile Tyr Thr	
100 105 110	
TTA CGT GAA CTG GTT CAC AGT TTA GGT ATC TCT AAA CCA ACA ATT GTA	384
Leu Arg Glu Leu Val His Ser Leu Gly Ile Ser Lys Pro Thr Ile Val	
115 120 125	
TTT AGT TCT AAA AAA GGC TTA GAT AAA GTT ATA ACA GTA CAG AAA ACA	432
Phe Ser Ser Lys Lys Gly Leu Asp Lys Val Ile Thr Val Gln Lys Thr	
130 135 140	
GTA ACT ACT ATT AAA ACC ATT GTT ATA CTA GAT AGC AAA GTT GAT TAT	480
Val Thr Thr Ile Lys Thr Ile Val Ile Leu Asp Ser Lys Val Asp Tyr	
145 150 155 160	
CGA GGA TAT CAA TGT CTG GAC ACC TTT ATA AAA AGA AAC ACT CCA CCA	528
Arg Gly Tyr Gln Cys Leu Asp Thr Phe Ile Lys Arg Asn Thr Pro Pro	
165 170 175	
GGT TTT CAA GCA TCC AGT TTC AAA ACT GTG GAA GTT GAC CGT AAA GAA	576
Gly Phe Gln Ala Ser Ser Phe Lys Thr Val Glu Val Asp Arg Lys Glu	
180 185 190	
CAA GTT GCT CTT ATA ATG AAC TCT TCG GGT TCT ACC GGT TTG CCA AAA	624
Gln Val Ala Leu Ile Met Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys	
195 200 205	
GGC GTA CAA CTT ACT CAC GAA AAT ACA GTC ACT AGA TTT TCT CAT GCT	672

-8/43-

Gly	Val	Gln	Leu	Thr	His	Glu	Asn	Thr	Val	Thr	Arg	Phe	Ser	His	Ala	
210						215					220					
AGA	GAT	CCG	ATT	TAT	GGT	AAC	CAA	GTT	TCA	CCA	GGC	ACC	GCT	GTT	TTA	720
Arg	Asp	Pro	Ile	Tyr	Gly	Asn	Gln	Val	Ser	Pro	Gly	Thr	Ala	Val	Leu	
225					230					235					240	
ACT	GTC	GTT	CCA	TTC	CAT	CAT	GGT	TTT	GGT	ATG	TTC	ACT	ACT	CTA	GGG	768
Thr	Val	Val	Pro	Phe	His	His	Gly	Phe	Gly	Met	Phe	Thr	Thr	Leu	Gly	
				245					250					255		
TAT	TTA	ATT	TGT	GGT	TTT	CGT	GTT	GTA	ATG	TTA	ACA	AAA	TTC	GAT	GAA	816
Tyr	Leu	Ile	Cys	Gly	Phe	Arg	Val	Val	Met	Leu	Thr	Lys	Phe	Asp	Glu	
			260					265					270			
GAA	ACA	TTT	TTA	AAA	ACT	CTA	CAA	GAT	TAT	AAA	TGT	ACA	AGT	GTT	ATT	864
Glu	Thr	Phe	Leu	Lys	Thr	Leu	Gln	Asp	Tyr	Lys	Cys	Thr	Ser	Val	Ile	
		275					280					285				
CTT	GTA	CCG	ACC	TTG	TTT	GCA	ATT	CTC	AAC	AAA	AGT	GAA	TTA	CTC	AAT	912
Leu	Val	Pro	Thr	Leu	Phe	Ala	Ile	Leu	Asn	Lys	Ser	Glu	Leu	Leu	Asn	
	290					295					300					
AAA	TAC	GAT	TTG	TCA	AAT	TTA	GTT	GAG	ATT	GCA	TCT	GGC	GGA	GCA	CCT	960
Lys	Tyr	Asp	Leu	Ser	Asn	Leu	Val	Glu	Ile	Ala	Ser	Gly	Gly	Ala	Pro	
305					310					315					320	
TTA	TCA	AAA	GAA	GTT	GGT	GAA	GCT	GTT	GCT	AGA	CGC	TTT	AAT	CTT	CCC	1008
Leu	Ser	Lys	Glu	Val	Gly	Glu	Ala	Val	Ala	Arg	Arg	Phe	Asn	Leu	Pro	
				325					330					335		
GGT	GTT	CGT	CAA	GGT	TAT	GGT	TTA	ACA	GAA	ACA	ACA	TCT	GCC	ATT	ATT	1056
Gly	Val	Arg		Gly	Tyr	Gly	Leu	Thr	Glu	Thr	Thr	Ser	Ala	Ile	Ile	
			340					345					350			
ATT	ACA	CCA	GAA	GGA	GAC	GAT	AAA	CCA	GGA	GCT	TCT	GGA	AAA	GTC	GTG	1104
Ile	Thr	Pro	Glu	Gly	Asp	Asp	Lys	Pro	Gly	Ala	Ser	Gly	Lys	Val	Val	
		355					360					365				
CCG	TTG	TTT	AAA	GCA	AAA	GTT	ATT	GAT	CTT	GAT	ACC	AAA	AAA	TCT	TTA	1152
Pro	Leu	Phe	Lys	Ala	Lys	Val	Ile	Asp	Leu	Asp	Thr	Lys	Lys	Ser	Leu	
		370				375					380					
GGT	CCT	AAC	AGA	CGT	GGA	GAA	GTT	TGT	GTT	AAA	GGA	CCT	ATG	CTT	ATG	1200
Gly	Pro	Asn	Arg	Arg	Gly	Glu	Val	Cys	Val	Lys	Gly	Pro	Met	Leu	Met	
					390					395				400		
AAA	GGT	TAT	GTA	AAT	AAT	CCA	GAA	GCA	ACA	AAA	GAA	CTT	ATT	GAC	GAA	1248
Lys	Gly	Tyr	Val	Asn	Asn	Pro	Glu	Ala	Thr	Lys	Glu	Leu	Ile	Asp	Glu	
				405					410					415		
GAA	GGT	TGG	CTG	CAC	ACC	GGA	GAT	ATT	GGA	TAT	TAT	GAT	GAA	GAA	AAA	1296
Glu	Gly	Trp	Leu	His	Thr	Gly	Asp	Ile	Gly	Tyr	Tyr	Asp	Glu	Glu	Lys	
			420					425					430			
CAT	TTC	TTT	ATT	GTC	GAT	CGT	TTG	AAG	TCT	TTA	ATC	AAA	TAC	AAA	GGA	1344
His	Phe	Phe	Ile	Val	Asp	Arg	Leu	Lys	Ser	Leu	Ile	Lys	Tyr	Lys	Gly	
		435					440					445				
TAC	CAA	GTA	CCA	CCT	GCC	GAA	TTA	GAA	TCC	GTT	CTT	TTG	CAA	CAT	CCA	1392
Tyr	Gln	Val	Pro	Pro	Ala	Glu	Leu	Glu	Ser	Val	Leu	Leu	Gln	His	Pro	

-9/43-

450	455	460	
TCT ATC TTT GAT GCT GGT GTT GCC GGC GTT CCT GAT CCT GTA GCT GGC			1440
Ser Ile Phe Asp Ala Gly Val Ala Gly Val Pro Asp Pro Val Ala Gly			
465	470	475	480
GAG CTT CCA GGA GCC GTT GTT GTA CTG GAA AGC GGA AAA AAT ATG ACC			1488
Glu Leu Pro Gly Ala Val Val Val Leu Glu Ser Gly Lys Asn Met Thr			
	485	490	495
GAA AAA GAA GTA ATG GAT TAT GTT GCA AGT CAA GTT TCA AAT GCA AAA			1536
Glu Lys Glu Val Met Asp Tyr Val Als Ser Gln Val Ser Asn Ala Lys			
	500	505	510
CGT TTA CGT GGT GGT GTT CGT TTT GTG GAT GAA GTA CCT AAA GGT CTT			1584
Arg Leu Arg Gly Gly Val Arg Phe Val Asp Glu Val Pro Lys Gly Leu			
	515	520	525
ACT GGA AAA ATT GAC GGC AGA GCA ATT AGA GAA ATC CTT AAG AAA CCA			1632
Thr Gly Lys Ile Asp Gly Arg Ala Ile Arg Glu Ile Leu Lys Lys Pro			
	530	535	540
GTT GCT AAG ATG			1644
Val Ala Lys Met			
545			

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1820 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
- (B) LOCATION: 1...1664
- (D) OTHER INFORMATION: Vargula (cypridina) luciferase

## (x) PUBLICATION INFORMATION:

- JP 3-30678 Osaka (Tsuji)
- (A) AUTHORS: Thompson *et al.*
- (C) JOURNAL: Proc. Natl. Acad. Sci. U.S.A.
- (D) VOLUME: 86
- (F) PAGES: 1326-1332
- (G) DATE: (1989)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATG AAG ATA ATA ATT CTG TCT GTT ATA TTG GCC TAC TGT GTC ACC GAC	48
Met Lys Ile Ile Ile Leu Ser Val Ile Leu Ala Tyr Cys Val Thr Asp	
1 5 10 15	
AAC TGT CAA GAT GCA TGT CCT GTA GAA GCG GAA CCG CCA TCA AGT ACA	96
Asn Cys Gln Asp Ala Cys Pro Val Glu Ala Glu Pro Pro Ser Ser Thr	
20 25 30	
CCA ACA GTT CCA ACT TCT TGT GAA GCT AAA GAA GGA GAA TGT ATA GAT	144
Pro Thr Val Pro Thr Ser Cys Glu Ala Lys Glu Gly Glu Cys Ile Asp	

-10/43-

35						40				45						
ACC Thr	AGA Arg	TGC Cys	GCA Ala	ACA Thr	TGT Cys	AAA Lys	CGA Arg	GAT Asp	ATA Ile	CTA Leu	TCA Ser	GAT Asp	GGA Gly	CTG Leu	TGT Cys	192
50																
GAA Glu	AAT Asn	AAA Lys	CCA Pro	GGG Gly	AAG Lys	ACA Thr	TGC Cys	TGT Cys	AGA Arg	ATG Met	TGC Cys	CAG Gln	TAT Tyr	GTG Val	ATT Ile	240
65																
GAA Glu	TGC Cys	AGA Arg	GTA Val	GAA Glu	GCA Ala	GCT Ala	GGT Gly	TAT Tyr	TTT Phe	AGA Arg	ACG Thr	TTT Phe	TAC Tyr	GGC Gly	AAA Lys	288
85																
AGA Arg	TTT Phe	AAT Asn	TTT Phe	CAG Gln	GAA Glu	CCT Pro	GGT Gly	AAA Lys	TAT Tyr	GTG Val	CTG Leu	GCT Ala	AGG Arg	GGA Gly	ACC Thr	336
100																
AAG Lys	GGT Gly	GGC Gly	GAT Asp	TGG Trp	TCT Ser	GTA Val	ACC Thr	CTC Leu	ACC Thr	ATG Met	GAG Glu	AAT Asn	CTA Leu	GAT Asp	GGA Gly	384
115																
CAG Gln	AAG Lys	GGA Gly	GCT Ala	GTG Val	CTG Leu	ACT Thr	AAG Lys	ACA Thr	ACA Thr	CTG Leu	GAG Glu	GTT Val	GCA Ala	GGA Gly	GAC Asp	432
130																
135																
140																
145																
GTA Val	ATA Ile	GAC Asp	ATT Ile	ACT Thr	CAA Gln	GCT Ala	ACT Thr	GCA Ala	GAT Asp	CCT Pro	ATC Ile	ACA Thr	GTT Val	AAC Asn	GGA Gly	480
150																
155																
160																
GGA Gly	GCT Ala	GAC Asp	CCA Pro	GTT Val	ATC Ile	GCT Ala	AAC Asn	CCG Pro	TTC Phe	ACA Thr	ATT Ile	GGT Gly	GAG Glu	GTG Val	ACC Thr	528
165																
170																
175																
ATT Ile	GCT Ala	GTT Val	GTT Val	GAA Glu	ATA Ile	CCG Pro	GGC Gly	TTC Phe	AAT Asn	ATC Ile	ACA Thr	GTC Val	ATC Ile	GAA Glu	TTC Phe	576
180																
185																
190																
TTT Phe	AAA Lys	CTA Leu	ATC Ile	GTG Val	ATT Ile	GAT Asp	ATT Ile	CTG Leu	GGA Gly	GGA Gly	AGA Arg	TCT Ser	GTC Val	AGA Arg	ATT Ile	624
195																
200																
205																
GCT Ala	CCA Pro	GAC Asp	ACA Thr	GCA Ala	AAC Asn	AAA Lys	GGA Gly	CTG Leu	ATA Ile	TCT Ser	GGT Gly	ATC Ile	TGT Cys	GGT Gly	AAT Asn	672
210																
215																
220																
CTG Leu	GAG Glu	ATG Met	AAT Asn	GAC Asp	GCT Ala	GAT Asp	GAC Asp	TTT Phe	ACT Thr	ACA Thr	GAT Asp	GCA Ala	GAT Asp	CAG Gln	CTG Leu	720
225																
230																
235																
240																
GCG Ala	ATC Ile	CAA Gln	CCC Pro	AAC Asn	ATA Ile	AAC Asn	AAA Lys	GAG Glu	TTC Phe	GAC Asp	GGC Gly	TGC Cys	CCA Pro	TTC Phe	TAT Tyr	768
245																
250																
255																
GGC Gly	AAT Asn	CCT Pro	TCT Ser	GAT Asp	ATC Ile	GAA Glu	TAC Tyr	TGC Cys	AAA Lys	GGT Gly	CTG Leu	ATG Met	GAG Glu	CCA Pro	TAC Tyr	816
260																
265																
270																
AGA Arg	GCT Ala	GTA Val	TGT Cys	CGT Arg	AAC Asn	AAT Asn	ATC Ile	AAC Asn	TTC Phe	TAC Tyr	TAT Tyr	TAC Tyr	ACT Thr	CTA Leu	TCC Ser	864
275																
280																
285																

-11/43-

TGT Cys 290	GCC Ala 290	TTC Phe 290	GCT Ala 290	TAC Tyr 290	TGT Cys 295	ATG Met 295	GGA Gly 295	GGA Gly 295	GAA Glu 295	GAA Glu 300	AGA Arg 300	GCT Ala 300	AAA Lys 300	CAC His 300	GTC Val 300	912
CTT Leu 305	TTC Phe 305	GAC Asp 305	TAT Tyr 305	GTT Val 305	GAG Glu 310	ACA Thr 310	TGC Cys 310	GCT Ala 310	GCG Ala 315	CCG Pro 315	GAA Glu 315	ACG Thr 315	AGA Arg 315	GGA Gly 320	ACG Thr 320	960
TGT Cys 325	GTT Val 325	TTA Leu 325	TCA Ser 325	GGA Gly 325	CAT His 325	ACT Thr 325	TTC Phe 325	TAT Tyr 325	GAC Asp 330	ACA Thr 330	TTC Phe 330	GAC Asp 330	AAA Lys 330	GCA Ala 335	AGA Arg 335	1008
TAT Tyr 340	CAA Gln 340	TTC Phe 340	CAG Gln 340	GGC Gly 340	CCA Pro 340	TGC Cys 340	AAG Lys 345	GAG Glu 345	ATT Ile 345	CTG Leu 345	ATG Met 345	GCC Ala 350	GCA Ala 350	GAC Asp 350	TGT Cys 350	1056
TAC Tyr 355	TGG Trp 355	AAC Asn 355	ACA Thr 355	TGG Trp 355	GAT Asp 355	GTA Val 360	AAG Lys 360	GTT Val 360	TCA Ser 360	CAT His 360	AGA Arg 365	GAC Asp 365	GTC Val 365	GAA Glu 365	TCA Ser 365	1104
TAC Tyr 370	ACT Thr 370	GAG Glu 370	GTA Val 370	GAG Glu 370	AAA Lys 375	GTA Val 375	ACA Thr 375	ATC Ile 375	AGG Arg 375	AAA Lys 380	CAG Gln 380	TCA Ser 380	ACT Thr 380	GTA Val 380	GTA Val 380	1152
GAT Asp 385	CTC Leu 385	ATT Ile 385	GTG Val 385	GAT Asp 390	GGC Gly 390	AAG Lys 390	CAG Gln 390	GTC Val 390	AAG Lys 395	GTT Val 395	GGA Gly 395	GGA Gly 395	GTG Val 395	GAT Asp 400	GTA Val 400	1200
TCT Ser 405	ATC Ile 405	CCG Pro 405	TAC Tyr 405	AGC Ser 405	TCT Ser 405	GAG Glu 405	AAC Asn 410	ACT Thr 410	TCC Ser 410	ATA Ile 410	TAC Tyr 410	TGG Trp 410	CAG Gln 415	GAT Asp 415	GGA Gly 415	1248
GAC Asp 420	ATC Ile 420	CTG Leu 420	ACG Thr 420	ACG Thr 420	GCC Ala 420	ATC Ile 420	CTA Leu 425	CCT Pro 425	GAA Glu 425	GCT Ala 425	CTT Leu 425	GTC Val 430	GTT Val 430	AAG Lys 430	TTC Phe 430	1296
AAC Asn 435	TTT Phe 435	AAG Lys 435	CAG Gln 435	CTC Leu 435	CTT Leu 435	GTA Val 440	GTT Val 440	CAT His 440	ATC Ile 440	AGA Arg 440	GAT Asp 445	CCA Pro 445	TTC Phe 445	GAT Asp 445	GCA Ala 445	1344
AAG Lys 450	ACA Thr 450	TGC Cys 450	GGC Gly 450	ATA Ile 450	TGT Cys 455	GGT Gly 455	AAC Asn 455	TAT Tyr 455	AAT Asn 455	CAA Gln 460	GAT Asp 460	TCA Ser 460	ACT Thr 460	GAT Asp 460	GAT Asp 460	1392
TTC Phe 465	TTT Phe 465	GAC Asp 465	GCA Ala 465	GAA Glu 465	GGA Gly 470	GCA Ala 470	TGC Cys 470	GCT Ala 470	CTA Leu 475	ACC Thr 475	CCC Pro 475	AAC Asn 475	CCC Pro 475	CCA Pro 480	GGA Gly 480	1440
TGT Cys 485	ACA Thr 485	GAG Glu 485	GAA Glu 485	CAG Gln 485	AAA Lys 485	CCA Pro 485	GAA Glu 490	GCT Ala 490	GAG Glu 490	CGA Arg 490	CTT Leu 490	TGC Cys 490	AAT Asn 495	AAT Asn 495	CTC Leu 495	1488
TTT Phe 500	GAT Asp 500	TCT Ser 500	TCT Ser 500	ATC Ile 500	GAC Asp 500	GAG Glu 505	AAA Lys 505	TGT Cys 505	AAT Asn 505	GTC Val 505	TGC Cys 510	TAC Tyr 510	AAG Lys 510	CCT Pro 510	GAC Asp 510	1536
CGG Arg 515	ATT Ile 515	GCC Ala 515	CGA Arg 515	TGT Cys 515	ATG Met 515	TAC Tyr 520	GAG Glu 520	TAT Tyr 520	TGC Cys 520	CTG Leu 520	AGG Arg 525	GGA Gly 525	CAA Gln 525	CAA Gln 525	GGA Gly 525	1584
TTT Phe 532	TGT Cys 532	GAC Asp 532	CAT His 532	GCT Ala 532	TGG Trp 532	GAG Glu 532	TTC Phe 532	AAG Lys 532	AAA Lys 532	GAA Glu 532	TGC Cys 532	TAC Tyr 532	ATA Ile 532	AAA Lys 532	CAT His 532	1632

-12/43-

530	535	540	
GGA GAC ACT CTA GAA GTA CCA CCT GAA TGT CAA TAA ACGTACAAAG			1678
Gly Asp Thr Leu Glu Val Pro Pro Glu Cys Gln			
545	550	555	
ATACAGAAGC TAAGGCTACT ACAGCAGAAG ATAAAAAAGA AACTGTAGTT CCTTCAAAAA			1738
CCGTGTATTT TATGTACTCA TTGTTTAATT AGAGCAAAAT AAATTGTTAT TATCATAACT			1798
TAAACTAAAA AAAAAAAAAA AA			1820

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 958 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (iii) HYPOTHETICAL: NO

## (iv) ANTISENSE: NO

## (v) FRAGMENT TYPE:

## (vi) ORIGINAL SOURCE:

## (ix) FEATURE:

## (A) NAME/KEY: Coding Sequence

## (B) LOCATION: 115...702

## (D) OTHER INFORMATION: apoaequorin-encoding gene

## (x) PUBLICATION INFORMATION:

PATENT NO.: 5,093,240

(A) AUTHORS: Inouye *et al.*

(C) JOURNAL: Proc. Natl. Acad. Sci. U.S.A.

(D) VOLUME: 82

(F) PAGES: 3154-3158

(G) DATE: (1985)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGGGGGGGGG GGGGGGGGGG GGGGGGGGGG GGGGAATGCAA TTCATCTTTG CATCAAAGAA	60
TTACATCAAAA TCTCTAGTTG ATCAACTAAA TTGTCTCGAC AACAACAAGC AAAC ATG	117
	Met
	1
ACA AGC AAA CAA TAC TCA GTC AAG CTT ACA TCA GAC TTC GAC AAC CCA	165
Thr Ser Lys Gln Tyr Ser Val Lys Leu Thr Ser Asp Phe Asp Asn Pro	
5 10 15	
AGA TGG ATT GGA CGA CAC AAG CAT ATG TTC AAT TTC CTT GAT GTC AAC	213
Arg Trp Ile Gly Arg His Lys His Met Phe Asn Phe Leu Asp Val Asn	
20 25 30	
CAC AAT GGA AAA ATC TCT CTT GAC GAG ATG GTC TAC AAG GCA TCT GAT	261
His Asn Gly Lys Ile Ser Leu Asp Glu Met Val Tyr Lys Ala Ser Asp	
35 40 45	
ATT GTC ATC AAT AAC CTT GGA GCA ACA CCT GAG CAA GCC AAA CGA CAC	309
Ile Val Ile Asn Asn Leu Gly Ala Thr Pro Glu Gln Ala Lys Arg His	
50 55 60 65	

-13/43-

AAA GAT GCT GTA GAA GCC TTC TTC GGA GGA GCT GGA ATG AAA TAT GGT	357
Lys Asp Ala Val Glu Ala Phe Phe Gly Gly Ala Gly Met Lys Tyr Gly	
70 75 80	
GTG GAA ACT GAT TGG CCT GCA TAT ATT GAA GGA TGG AAA AAA TTG GCT	405
Val Glu Thr Asp Trp Pro Ala Tyr Ile Glu Gly Trp Lys Lys Leu Ala	
85 90 95	
ACT GAT GAA TTG GAG AAA TAC GCC AAA AAC GAA CCA ACG CTC ATC CGT	453
Thr Asp Glu Leu Glu Lys Tyr Ala Lys Asn Glu Pro Thr Leu Ile Arg	
100 105 110	
ATA TGG GGT GAT GCT TTG TTT GAT ATC GTT GAC AAA GAT CAA AAT GGA	501
Ile Trp Gly Asp Ala Leu Phe Asp Ile Val Asp Lys Asp Gln Asn Gly	
115 120 125	
GCC ATT ACA CTG GAT GAA TGG AAA GCA TAC ACC AAA GCT GCT GGT ATC	549
Ala Ile Thr Leu Asp Glu Trp Lys Ala Tyr Thr Lys Ala Ala Gly Ile	
130 135 140 145	
ATC CAA TCA TCA GAA GAT TGC GAG GAA ACA TTC AGA GTG TGC GAT ATT	597
Ile Gln Ser Ser Glu Asp Cys Glu Glu Thr Phe Arg Val Cys Asp Ile	
150 155 160	
GAT GAA AGT GGA CAA CTC GAT GTT GAT GAG ATG ACA AGA CAA CAT TTA	645
Asp Glu Ser Gly Gln Leu Asp Val Asp Glu Met Thr Arg Gln His Leu	
165 170 175	
GGA TTT TGG TAC ACC ATG GAT CCT GCT TGC GAA AAG CTC TAC GGT GGA	693
Gly Phe Trp Tyr Thr Met Asp Pro Ala Cys Glu Lys Leu Tyr Gly Gly	
180 185 190	
GCT GTC CCC TAAGAAGCTC TACGGTGGTG ATGCACCCTA GGAAGATGAT GTGATTTTGA	752
Ala Val Pro	
195	
ATAAAACACT GATGAATTCA ATCAAAATTT TCCTAAATTTT TGAACGATTT CAATCGTTTG	812
TGTTGATTTT TGTAATTAGG AACAGATTAA ATCGAATGAT TAGTTGTTTT TTTAATCAAC	872
AGAACTTACA AATCGAAAAA GTAAAAAAA AAAAAAAA AAAAAAAA AAAAAAAA	932
AAAAAAA AAAAAAAA AAAAAA	958

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 591 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (iii) HYPOTHETICAL: NO

## (iv) ANTISENSE: NO

## (v) FRAGMENT TYPE:

## (vi) ORIGINAL SOURCE:

## (ix) FEATURE:

(A) NAME/KEY: Coding Sequence

(B) LOCATION: 1...588

(D) OTHER INFORMATION: Recombinant Aequorin AEQ1

## (x) PUBLICATION INFORMATION:

-14/43-

(A) AUTHORS: Prasher et al.  
 (B) TITLE: Sequence Comparisons of Complementary  
 DNAs Encoding Aequorin Isotypes  
 (C) JOURNAL: Biochemistry  
 (D) VOLUME: 26  
 (F) PAGES: 1326-1332  
 (G) DATE: 1987

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATG ACC AGC GAA CAA TAC TCA GTC AAG CTT ACA CCA GAC TTC GAC AAC	48
Met Thr Ser Glu Gln Tyr Ser Val Lys Leu Thr Pro Asp Phe Asp Asn	
1 5 10 15	
CCA AAA TGG ATT GGA CGA CAC AAG CAC ATG TTT AAT TTT CTT GAT GTC	96
Pro Lys Trp Ile Gly Arg His Lys His Met Phe Asn Phe Leu Asp Val	
20 25 30	
AAC CAC AAT GGA AGG ATC TCT CTT GAC GAG ATG GTC TAC AAG GCG TCC	144
Asn His Asn Gly Arg Ile Ser Leu Asp Glu Met Val Tyr Lys Ala Ser	
35 40 45	
GAT ATT GTT ATA AAC AAT CTT GGA GCA ACA CCT GAA CAA GCC AAA CGT	192
Asp Ile Val Ile Asn Asn Leu Gly Ala Thr Pro Glu Gln Ala Lys Arg	
50 55 60	
CAC AAA GAT GCT GTA GAA GCC TTC TTC GGA GGA GCT GGA ATG AAA TAT	240
His Lys Asp Ala Val Glu Ala Phe Phe Gly Gly Ala Gly Met Lys Tyr	
65 70 75 80	
GGT GTA GAA ACT GAA TGG CCT GAA TAC ATC GAA GGA TGG AAA AGA CTG	288
Gly Val Glu Thr Glu Trp Pro Glu Tyr Ile Glu Gly Trp Lys Arg Leu	
85 90 95	
GCT TCC GAG GAA TTG AAA AGG TAT TCA AAA AAC CAA ATC ACA CTT ATT	336
Ala Ser Glu Glu Leu Lys Arg Tyr Ser Lys Asn Gln Ile Thr Leu Ile	
100 105 110	
CGT TTA TGG GGT GAT GCA TTG TTC GAT ATC ATT GAC AAA GAC CAA AAT	384
Arg Leu Trp Gly Asp Ala Leu Phe Asp Ile Ile Asp Lys Asp Gln Asn	
115 120 125	
GGA GCT ATT TCA CTG GAT GAA TGG AAA GCA TAC ACC AAA TCT GAT GGC	432
Gly Ala Ile Ser Leu Asp Glu Trp Lys Ala Tyr Thr Lys Ser Asp Gly	
130 135 140	
ATC ATC CAA TCG TCA GAA GAT TGC GAG GAA ACA TTC AGA GTG TGC GAT	480
Ile Ile Gln Ser Ser Glu Asp Cys Glu Glu Thr Phe Arg Val Cys Asp	
145 150 155 160	
ATT GAT GAA AGT GGA CAG CTC GAT GTT GAT GAG ATG ACA AGA CAA CAT	528
Ile Asp Glu Ser Gly Gln Leu Asp Val Asp Glu Met Thr Arg Gln His	
165 170 175	
TTA GGA TTT TGG TAC ACC ATG GAT CCT GCT TGC GAA AAG CTC TAC GGT	576
Leu Gly Phe Trp Tyr Thr Met Asp Pro Ala Cys Glu Lys Leu Tyr Gly	
180 185 190	
GGA GCT GTC CCC TAA	591
Gly Ala Val Pro *	
195	



-15/43-

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 591 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (iii) HYPOTHETICAL: NO

## (iv) ANTISENSE: NO

## (v) FRAGMENT TYPE:

## (vi) ORIGINAL SOURCE:

## (ix) FEATURE:

## (A) NAME/KEY: Coding Sequence

## (B) LOCATION: 1...588

## (D) OTHER INFORMATION: Recombinant Aequorin AEQ2

## (x) PUBLICATION INFORMATION:

## (A) AUTHORS: Prasher et al.

## (B) TITLE: Sequence Comparisons of Complementary DNAs Encoding Aequorin Isotypes

## (C) JOURNAL: Biochemistry

## (D) VOLUME: 26

## (F) PAGES: 1326-1332

## (G) DATE: 1987

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATG ACC AGC GAA CAA TAC TCA GTC AAG CTT ACA TCA GAC TTC GAC AAC	48
Met Thr Ser Glu Gln Tyr Ser Val Lys Leu Thr Ser Asp Phe Asp Asn	
1 5 10 15	
CCA AGA TGG ATT GGA CGA CAC AAG CAT ATG TTC AAT TTC CTT GAT GTC	96
Pro Arg Trp Ile Gly Arg His Lys His Met Phe Asn Phe Leu Asp Val	
20 25 30	
AAC CAC AAT GGA AAA ATC TCT CTT GAC GAG ATG GTC TAC AAG GCA TCT	144
Asn His Asn Gly Lys Ile Ser Leu Asp Glu Met Val Tyr Lys Ala Ser	
35 40 45	
GAT ATT GTC ATC AAT AAC CTT GGA GCA ACA CCT GAG CAA GCC AAA CGA	192
Asp Ile Val Ile Asn Asn Leu Gly Ala Thr Pro Glu Gln Ala Lys Arg	
50 55 60	
CAC AAA GAT GCT GTA GAA GCC TTC TTC GGA GGA GCT GGA ATG AAA TAT	240
His Lys Asp Ala Val Glu Ala Phe Phe Gly Gly Ala Gly Met Lys Tyr	
65 70 75 80	
GGT GTG GAA ACT GAT TGG CCT GCA TAT ATT GAA GGA TGG AAA AAA TTG	288
Gly Val Glu Thr Asp Trp Pro Ala Tyr Ile Glu Gly Trp Lys Lys Leu	
85 90 95	
GCT ACT GAT GAA TTG GAG AAA TAC GCC AAA AAC GAA CCA ACG CTC ATC	336
Ala Thr Asp Glu Leu Glu Lys Tyr Ala Lys Asn Glu Pro Thr Leu Ile	
100 105 110	
CGT ATA TGG GGT GAT GCT TTG TTC GAT ATC GTT GAC AAA GAT CAA AAT	384
Arg Ile Trp Gly Asp Ala Leu Phe Asp Ile Val Asp Lys Asp Gln Asn	

-16/43-

115	120	125	
GGA GCC ATT ACA CTG GAT GAA TGG AAA GCA TAC ACC AAA GCT GCT GGT			432
Gly Ala Ile Thr Leu Asp Glu Trp Lys Ala Tyr Thr Lys Ala Ala Gly			
130	135	140	
ATC ATC CAA TCA TCA GAA GAT TGC GAG GAA ACA TTC AGA GTG TGC GAT			480
Ile Ile Gln Ser Ser Glu Asp Cys Glu Glu Thr Phe Arg Val Cys Asp			
145	150	155	160
ATT GAT GAA AGT GGA CAA CTC GAT GTT GAT GAG ATG ACA AGA CAA CAT			528
Ile Asp Glu Ser Gly Gln Leu Asp Val Asp Glu Met Thr Arg Gln His			
	165	170	175
TTA GGA TTT TGG TAC ACC ATG GAT CCT GCT TGC GAA AAG CTC TAC GGT			576
Leu Gly Phe Trp Tyr Thr Met Asp Pro Ala Cys Glu Lys Leu Tyr Gly			
	180	185	190
GGA GCT GTC CCC TAA			591
Gly Ala Val Pro *			
195			

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 591 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (iii) HYPOTHETICAL: NO

## (iv) ANTISENSE: NO

## (v) FRAGMENT TYPE:

## (vi) ORIGINAL SOURCE:

## (ix) FEATURE:

## (A) NAME/KEY: Coding Sequence

## (B) LOCATION: 1...588

## (D) OTHER INFORMATION: Recombinant Aequorin AEQ3

## (x) PUBLICATION INFORMATION:

## (A) AUTHORS: Prasher et al.

## (B) TITLE: Sequence Comparisons of Complementary DNAs Encoding Aequorin Isoforms

## (C) JOURNAL: Biochemistry

## (D) VOLUME: 26

## (F) PAGES: 1326-1332

## (G) DATE: 1987

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATG ACC AGC GAA CAA TAC TCA GTC AAG CTT ACA TCA GAC TTC GAC AAC	48
Met Thr Ser Glu Gln Tyr Ser Val Lys Leu Thr Ser Asp Phe Asp Asn	
1	5
CCA AGA TGG ATT GGA CGA CAC AAG CAT ATG TTC AAT TTC CTT GAT GTC	96
Pro Arg Trp Ile Gly Arg His Lys His Met Phe Asn Phe Leu Asp Val	
20	25
	30

-17/43-

AAC CAC AAT GGA AAA ATC TCT CTT GAC GAG ATG GTC TAC AAG GCA TCT Asn His Asn Gly Lys Ile Ser Leu Asp Glu Met Val Tyr Lys Ala Ser 35 40 45	144
GAT ATT GTC ATC AAT AAC CTT GGA GCA ACA CCT GAG CAA GCC AAA CGA Asp Ile Val Ile Asn Asn Leu Gly Ala Thr Pro Glu Gln Ala Lys Arg 50 55 60	192
CAC AAA GAT GCT GTA GGA GAC TTC TTC GGA GGA GCT GGA ATG AAA TAT His Lys Asp Ala Val Gly Asp Phe Phe Gly Gly Ala Gly Met Lys Tyr 65 70 75 80	240
GGT GTG GAA ACT GAT TGG CCT GCA TAC ATT GAA GGA TGG AAA AAA TTG Gly Val Glu Thr Asp Trp Pro Ala Tyr Ile Glu Gly Trp Lys Lys Leu 85 90 95	288
GCT ACT GAT GAA TTG GAG AAA TAC GCC AAA AAC GAA CCA ACG CTC ATC Ala Thr Asp Glu Leu Glu Lys Tyr Ala Lys Asn Glu Pro Thr Leu Ile 100 105 110	336
CGT ATA TGG GGT GAT GCT TTG TTC GAT ATC GTT GAC AAA GAT CAA AAT Arg Ile Trp Gly Asp Ala Leu Phe Asp Ile Val Asp Lys Asp Gln Asn 115 120 125	384
GGA GCC ATT ACA CTG GAT GAA TGG AAA GCA TAC ACC AAA GCT GCT GGT Gly Ala Ile Thr Leu Asp Glu Trp Lys Ala Tyr Thr Lys Ala Ala Gly 130 135 140	432
ATC ATC CAA TCA TCA GAA GAT TGC GAG GAA ACA TTC AGA GTG TGC GAT Ile Ile Gln Ser Ser Glu Asp Cys Glu Glu Thr Phe Arg Val Cys Asp 145 150 155 160	480
ATT GAT GAA AAT GGA CAA CTC GAT GTT GAT GAG ATG ACA AGA CAA CAT Ile Asp Glu Asn Gly Gln Leu Asp Val Asp Glu Met Thr Arg Gln His 165 170 175	528
TTA GGA TTT TGG TAC ACC ATG GAT CCT GCT TGC GAA AAG CTC TAC GGT Leu Gly Phe Trp Tyr Thr Met Asp Pro Ala Cys Glu Lys Leu Tyr Gly 180 185 190	576
GGA GCT GTC CCC TAA Gly Ala Val Pro *	591
195	

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 567 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (iii) HYPOTHETICAL: NO

## (iv) ANTISENSE: NO

## (v) FRAGMENT TYPE:

## (vi) ORIGINAL SOURCE:

## (ix) FEATURE:

## (A) NAME/KEY: Coding Sequence

## (B) LOCATION: 1...567

-18/43-

(D) OTHER INFORMATION: Aequorin photoprotein

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Charbonneau et al.  
 (B) TITLE: Amino acid sequence of the calcium-dependent photoprotein aequorin  
 (C) JOURNAL: Am. Chem. Soc.  
 (D) VOLUME: 24  
 (E) ISSUE: 24  
 (F) PAGES: 6762-6771  
 (G) DATE: 1985

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GTC AAG CTT ACA CCA GAC TTC GAC AAC CCA AAA TGG ATT GGA CGA CAC	48
Val Lys Leu Thr Pro Asp Phe Asp Asn Pro Lys Trp Ile Gly Arg His	
1 5 10 15	
AAG CAC ATG TTT AAT TTT CTT GAT GTC AAC CAC AAT GGA AGG ATC TCT	96
Lys His Met Phe Asn Phe Leu Asp Val Asn His Asn Gly Arg Ile Ser	
20 25 30	
CTT GAC GAG ATG GTC TAC AAG GCG TCC GAT ATT GTT ATA AAC AAT CTT	144
Leu Asp Glu Met Val Tyr Lys Ala Ser Asp Ile Val Ile Asn Asn Leu	
35 40 45	
GGA GCA ACA CCT GAA CAA GCC AAA CGT CAC AAA GAT GCT GTA GAA GCC	192
Gly Ala Thr Pro Glu Gln Ala Lys Arg His Lys Asp Ala Val Glu Ala	
50 55 60	
TTC TTC GGA GGA GCT GCA ATG AAA TAT GGT GTA GAA ACT GAA TGG CCT	240
Phe Phe Gly Gly Ala Ala Met Lys Tyr Gly Val Glu Thr Glu Trp Pro	
65 70 75 80	
GAA TAC ATC GAA GGA TGG AAA AGA CTG GCT TCC GAG GAA TTG AAA AGG	288
Glu Tyr Ile Glu Gly Trp Lys Arg Leu Ala Ser Glu Glu Leu Lys Arg	
85 90 95	
TAT TCA AAA AAC CAA ATC ACA CTT ATT CGT TTA TGG GGT GAT GCA TTG	336
Tyr Ser Lys Asn Gln Ile Thr Leu Ile Arg Leu Trp Gly Asp Ala Leu	
100 105 110	
TTC GAT ATC ATT GAC AAA GAC CAA AAT GGA GCT ATT TCA CTG GAT GAA	384
Phe Asp Ile Ile Asp Lys Asp Gln Asn Gly Ala Ile Ser Leu Asp Glu	
115 120 125	
TGG AAA GCA TAC ACC AAA TCT GCT GGC ATC ATC CAA TCG TCA GAA GAT	432
Trp Lys Ala Tyr Thr Lys Ser Ala Gly Ile Ile Gln Ser Ser Glu Asp	
130 135 140	
TGC GAG GAA ACA TTC AGA GTG TGC GAT ATT GAT GAA AGT GGA CAG CTC	480
Cys Glu Glu Thr Phe Arg Val Cys Asp Ile Asp Glu Ser Gly Gln Leu	
145 150 155 160	
GAT GTT GAT GAG ATG ACA AGA CAA CAT TTA GGA TTT TGG TAC ACC ATG	528
Asp Val Asp Glu Met Thr Arg Gln His Leu Gly Phe Trp Tyr Thr Met	
165 170 175	
GAT CCT GCT TGC GAA AAG CTC TAC GGT GGA GCT GTC CCC	567
Asp Pro Ala Cys Glu Lys Leu Tyr Gly Gly Ala Val Pro	

-19/43-

180

185

## (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 588 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence

(B) LOCATION: 1...588

(D) OTHER INFORMATION: Aequorin mutant w/increased bioluminescence activity

## (x) PUBLICATION INFORMATION:

PATENT NO.: 5,360,728

## (K) RELEVANT RESIDUES IN SEQ ID NO: 10:

Asp 124 changed to Ser

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATG ACC AGC GAA CAA TAC TCA GTC AAG CTT ACA CCA GAC TTC GAC AAC	48
Met Thr Ser Glu Gln Tyr Ser Val Lys Leu Thr Pro Asp Phe Asn	
1 5 10 15	
CCA AAA TGG ATT GGA CGA CAC AAG CAC ATG TTT AAT TTT CTT GAT GTC	96
Pro Lys Trp Ile Gly Arg His Lys His Met Phe Asn Phe Leu Asp Val	
20 25 30	
AAC CAC AAT GGA AGG ATC TCT CTT GAC GAG ATG GTC TAC AAG GCG TCC	144
Asn His Asn Gly Arg Ile Ser Leu Asp Glu Met Val Tyr Lys Ala Ser	
35 40 45	
GAT ATT GTT ATA AAC AAT CTT GGA GCA ACA CCT GAA CAA GCC AAA CGT	192
Asp Ile Val Ile Asn Asn Leu Gly Ala Thr Pro Glu Gln Ala Lys Arg	
50 55 60	
CAC AAA GAT GCT GTA GAA GCC TTC TTC GGA GGA GCT GCA ATG AAA TAT	240
His Lys Asp Ala Val Glu Ala Phe Phe Gly Gly Ala Ala Met Lys Tyr	
65 70 75 80	
GGT GTA GAA ACT GAA TGG CCT GAA TAC ATC GAA GGA TGG AAA AGA CTG	288
Gly Val Glu Thr Glu Trp Pro Glu Tyr Ile Glu Gly Trp Lys Arg Leu	
85 90 95	
GCT TCC GAG GAA TTG AAA AGG TAT TCA AAA AAC CAA ATC ACA CTT ATT	336
Ala Ser Glu Glu Leu Lys Arg Tyr Ser Lys Asn Gln Ile Thr Leu Ile	
100 105 110	
CGT TTA TGG GGT GAT GCA TTG TTC GAT ATC ATT TCC AAA GAC CAA AAT	384
Arg Leu Trp Gly Asp Ala Leu Phe Asp Ile Ile Ser Lys Asp Gln Asn	

-20/43-

115	120	125	
GGA GCT ATT TCA CTG GAT GAA TGG AAA GCA TAC ACC AAA TCT GCT GGC			432
Gly Ala Ile Ser Leu Asp Glu Trp Lys Ala Tyr Thr Lys Ser Ala Gly			
130	135	140	
ATC ATC CAA TCG TCA GAA GAT TGC GAG GAA ACA TTC AGA GTG TGC GAT			480
Ile Ile Gln Ser Ser Glu Asp Cys Glu Glu Thr Phe Arg Val Cys Asp			
145	150	155	160
ATT GAT GAA AGT GGA CAG CTC GAT GTT GAT GAG ATG ACA AGA CAA CAT			528
Ile Asp Glu Ser Gly Gln Leu Asp Val Asp Glu Met Thr Arg Gln His			
165	170	175	
TTA GGA TTT TGG TAC ACC ATG GAT CCT GCT TGC GAA AAG CTC TAC GGT			576
Leu Gly Phe Trp Tyr Thr Met Asp Pro Ala Cys Glu Lys Leu Tyr Gly			
180	185	190	
GGA GCT GTC CCC			588
Gly Ala Val Pro			
195			

## (2) INFORMATION FOR SEQ ID NO:11:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 588 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (iii) HYPOTHETICAL: NO

## (iv) ANTISENSE: NO

## (v) FRAGMENT TYPE:

## (vi) ORIGINAL SOURCE:

## (ix) FEATURE:

## (A) NAME/KEY: Coding Sequence

## (B) LOCATION: 1...588

(D) OTHER INFORMATION: Recombinant site-directed Aequorin mutant  
w/increased biolum. activity

## (x) PUBLICATION INFORMATION:

PATENT NO.: 5,360,728

## (K) RELEVANT RESIDUES IN SEQ ID NO: 11:

Glu 135 changed to Ser

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ATG ACC AGC GAA CAA TAC TCA GTC AAG CTT ACA CCA GAC TTC GAC AAC	48
Met Thr Ser Glu Gln Tyr Ser Val Lys Leu Thr Pro Asp Phe Asp Asn	
1 5 10 15	
CCA AAA TGG ATT GGA CGA CAC AAG CAC ATG TTT AAT TTT CTT GAT GTC	96
Pro Lys Trp Ile Gly Arg His Lys His Met Phe Asn Phe Leu Asp Val	
20 25 30	
AAC CAC AAT GGA AGG ATC TCT CTT GAC GAG ATG GTC TAC AAG GCG TCC	144
Asn His Asn Gly Arg Ile Ser Leu Asp Glu Met Val Tyr Lys Ala Ser	

-21/43-

35					40					45						
GAT	ATT	GTT	ATA	AAC	AAT	CTT	GGA	GCA	ACA	CCT	GAA	CAA	GCC	AAA	CGT	192
Asp	Ile	Val	Ile	Asn	Asn	Leu	Gly	Ala	Thr	Pro	Glu	Gln	Ala	Lys	Arg	
50						55					60					
CAC	AAA	GAT	GCT	GTA	GAA	GCC	TTC	TTC	GGA	GGA	GCT	GCA	ATG	AAA	TAT	240
His	Lys	Asp	Ala	Val	Glu	Ala	Phe	Phe	Gly	Gly	Ala	Ala	Met	Lys	Tyr	
65					70				75						80	
GGT	GTA	GAA	ACT	GAA	TGG	CCT	GAA	TAC	ATC	GAA	GGA	TGG	AAA	AGA	CTG	288
Gly	Val	Glu	Thr	Glu	Trp	Pro	Glu	Tyr	Ile	Glu	Gly	Trp	Lys	Arg	Leu	
				85					90					95		
GCT	TCC	GAG	GAA	TTG	AAA	AGG	TAT	TCA	AAA	AAC	CAA	ATC	ACA	CTT	ATT	336
Ala	Ser	Glu	Glu	Leu	Lys	Arg	Tyr	Ser	Lys	Asn	Gln	Ile	Thr	Leu	Ile	
			100					105					110			
CGT	TTA	TGG	GGT	GAT	GCA	TTG	TTC	GAT	ATC	ATT	TCC	AAA	GAC	CAA	AAT	384
Arg	Leu	Trp	Gly	Asp	Ala	Leu	Phe	Asp	Ile	Ile	Ser	Lys	Asp	Gln	Asn	
	115					120						125				
GGA	GCT	ATT	TCA	CTG	GAT	TCA	TGG	AAA	GCA	TAC	ACC	AAA	TCT	GCT	GGC	432
Gly	Ala	Ile	Ser	Leu	Asp	Ser	Trp	Lys	Ala	Tyr	Thr	Lys	Ser	Ala	Gly	
	130				135						140					
ATC	ATC	CAA	TCG	TCA	GAA	GAT	TGC	GAG	GAA	ACA	TTC	AGA	GTG	TGC	GAT	480
Ile	Ile	Gln	Ser	Ser	Glu	Asp	Cys	Glu	Glu	Thr	Phe	Arg	Val	Cys	Asp	
145					150				155						160	
ATT	GAT	GAA	AGT	GGA	CAG	CTC	GAT	GTT	GAT	GAG	ATG	ACA	AGA	CAA	CAT	528
Ile	Asp	Glu	Ser	Gly	Gln	Leu	Asp	Val	Asp	Glu	Met	Thr	Arg	Gln	His	
				165				170						175		
TTA	GGA	TTT	TGG	TAC	ACC	ATG	GAT	CCT	GCT	TGC	GAA	AAG	CTC	TAC	GGT	576
Leu	Gly	Phe	Trp	Tyr	Thr	Met	Asp	Pro	Ala	Cys	Glu	Lys	Leu	Tyr	Gly	
			180					185					190			
GGA	GCT	GTC	CCC													588
Gly	Ala	Val	Pro													
			195													

## (2) INFORMATION FOR SEQ ID NO:12:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 588 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (iii) HYPOTHETICAL: NO

## (iv) ANTISENSE: NO

## (v) FRAGMENT TYPE:

## (vi) ORIGINAL SOURCE:

## (ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
- (B) LOCATION: 1...588
- (D) OTHER INFORMATION: Recombinant site-directed  
Aequorin mutant w/increased biolum. activity

-22/43-

(x) PUBLICATION INFORMATION:  
PATENT NO.: 5,360,728

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATG ACC AGC GAA CAA TAC TCA GTC AAG CTT ACA CCA GAC TTC GAC AAC	48
Met Thr Ser Glu Gln Tyr Ser Val Lys Leu Thr Pro Asp Phe Asp Asn	
1 5 10 15	
CCA AAA TGG ATT GGA CGA CAC AAG CAC ATG TTT AAT TTT CTT GAT GTC	96
Pro Lys Trp Ile Gly Arg His Lys His Met Phe Asn Phe Leu Asp Val	
20 25 30	
AAC CAC AAT GGA AGG ATC TCT CTT GAC GAG ATG GTC TAC AAG GCG TCC	144
Asn His Asn Gly Arg Ile Ser Leu Asp Glu Met Val Tyr Lys Ala Ser	
35 40 45	
GAT ATT GTT ATA AAC AAT CTT GGA GCA ACA CCT GAA CAA GCC AAA CGT	192
Asp Ile Val Ile Asn Asn Leu Gly Ala Thr Pro Glu Gln Ala Lys Arg	
50 55 60	
CAC AAA GAT GCT GTA GAA GCC TTC TTC GGA GGA GCT GCA ATG AAA TAT	240
His Lys Asp Ala Val Glu Ala Phe Phe Gly Gly Ala Ala Met Lys Tyr	
65 70 75 80	
GGT GTA GAA ACT GAA TGG CCT GAA TAC ATC GAA GGA TGG AAA AGA CTG	288
Gly Val Glu Thr Glu Trp Pro Glu Tyr Ile Glu Gly Trp Lys Arg Leu	
85 90 95	
GCT TCC GAG GAA TTG AAA AGG TAT TCA AAA AAC CAA ATC ACA CTT ATT	336
Ala Ser Glu Glu Leu Lys Arg Tyr Ser Lys Asn Gln Ile Thr Leu Ile	
100 105 110	
CGT TTA TGG GGT GAT GCA TTG TTC GAT ATC ATT TCC AAA GAC CAA AAT	384
Arg Leu Trp Gly Asp Ala Leu Phe Asp Ile Ile Ser Lys Asp Gln Asn	
115 120 125	
GCA GCT ATT TCA CTG GAT GAA TGG AAA GCA TAC ACC AAA TCT GCT GGC	432
Ala Ala Ile Ser Leu Asp Glu Trp Lys Ala Tyr Thr Lys Ser Ala Gly	
130 135 140	
ATC ATC CAA TCG TCA GAA GAT TGC GAG GAA ACA TTC AGA GTG TGC GAT	480
Ile Ile Gln Ser Ser Glu Asp Cys Glu Glu Thr Phe Arg Val Cys Asp	
145 150 155 160	
ATT GAT GAA AGT GGA CAG CTC GAT GTT GAT GAG ATG ACA AGA CAA CAT	528
Ile Asp Glu Ser Gly Gln Leu Asp Val Asp Glu Met Thr Arg Gln His	
165 170 175	
TTA GGA TTT TGG TAC ACC ATG GAT CCT GCT TGC GAA AAG CTC TAC GGT	576
Leu Gly Phe Trp Tyr Thr Met Asp Pro Ala Cys Glu Lys Leu Tyr Gly	
180 185 190	
GGA GCT GTC CCC	588
Gly Ala Val Pro	
195	



-23/43-

## (2) INFORMATION FOR SEQ ID NO:13:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 567 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
- (B) LOCATION: 1...567
- (D) OTHER INFORMATION: Recombinant apoaeguorin (AQUALITE®)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTC	AAG	CTT	ACA	CCA	GAC	TTC	GAC	AAC	CCA	AAA	TGG	ATT	GGA	CGA	CAC	48
Val	Lys	Leu	Thr	Pro	Asp	Phe	Asp	Asn	Pro	Lys	Trp	Ile	Gly	Arg	His	
1				5					10					15		
AAG	CAC	ATG	TTT	AAT	TTT	CTT	GAT	GTC	AAC	CAC	AAT	GGA	AGG	ATC	TCT	96
Lys	His	Met	Phe	Asn	Phe	Leu	Asp	Val	Asn	His	Asn	Gly	Arg	Ile	Ser	
			20					25					30			
CTT	GAC	GAG	ATG	GTC	TAC	AAG	GCG	TCC	GAT	ATT	GTT	ATA	AAC	AAT	CTT	144
Leu	Asp	Glu	Met	Val	Tyr	Lys	Ala	Ser	Asp	Ile	Val	Ile	Asn	Asn	Leu	
		35					40					45				
GGA	GCA	ACA	CCT	GAA	CAA	GCC	AAA	CGT	CAC	AAA	GAT	GCT	GTA	GAA	GCC	192
Gly	Ala	Thr	Pro	Glu	Gln	Ala	Lys	Arg	His	Lys	Asp	Ala	Val	Glu	Ala	
	50					55					60					
TTC	TTC	GGA	GGA	GCT	GGA	ATG	AAA	TAT	GGT	GTA	GAA	ACT	GAA	TGG	CCT	240
Phe	Phe	Gly	Gly	Ala	Gly	Met	Lys	Tyr	Gly	Val	Glu	Thr	Glu	Trp	Pro	
65					70				75					80		
GAA	TAC	ATC	GAA	GGA	TGG	AAA	AAA	CTG	GCT	TCC	GAG	GAA	TTG	AAA	AGG	288
Glu	Tyr	Ile	Glu	Gly	Trp	Lys	Lys	Leu	Ala	Ser	Glu	Glu	Leu	Lys	Arg	
			85					90						95		
TAT	TCA	AAA	AAC	CAA	ATC	ACA	CTT	ATT	CGT	TTA	TGG	GGT	GAT	GCA	TTG	336
Tyr	Ser	Lys	Asn	Gln	Ile	Thr	Leu	Ile	Arg	Leu	Trp	Gly	Asp	Ala	Leu	
			100					105					110			
TTC	GAT	ATC	ATT	GAC	AAA	GAC	CAA	AAT	GGA	GCT	ATT	CTG	TCA	GAT	GAA	384
Phe	Asp	Ile	Ile	Asp	Lys	Asp	Gln	Asn	Gly	Ala	Ile	Leu	Ser	Asp	Glu	
		115					120					125				
TGG	AAA	GCA	TAC	ACC	AAA	TCT	GAT	GGC	ATC	ATC	CAA	TCG	TCA	GAA	GAT	432
Trp	Lys	Ala	Tyr	Thr	Lys	Ser	Asp	Gly	Ile	Ile	Gln	Ser	Ser	Glu	Asp	
	130					135					140					
TGC	GAG	GAA	ACA	TTC	AGA	GTG	TGC	GAT	ATT	GAT	GAA	AGT	GGA	CAG	CTC	480
Cys	Glu	Glu	Thr	Phe	Arg	Val	Cys	Asp	Ile	Asp	Glu	Ser	Gly	Gln	Leu	
145					150					155				160		
GAT	GTT	GAT	GAG	ATG	ACA	AGA	CAA	CAT	TTA	GGA	TTT	TGG	TAC	ACC	ATG	528
Asp	Val	Asp	Glu	Met	Thr	Arg	Gln	His	Leu	Gly	Phe	Trp	Tyr	Thr	Met	
				165					170					175		

-24/43-

GAT CCT GCT TGC GAA AAG CTC TAC GGT GGA GCT GTC CCC  
 Asp Pro Ala Cys Glu Lys Leu Tyr Gly Gly Ala Val Pro  
 180 185

567

## (2) INFORMATION FOR SEQ ID NO:14:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 236 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (x) PUBLICATION INFORMATION:

PATENT NO.: 5,484,723

## (ix) FEATURE:

(D) OTHER INFORMATION: Vibrio fisheri Flavin reductase

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Met Pro Ile Asn Cys Lys Val Lys Ser Ile Glu Pro Leu Ala Cys Asn  
 1 5 10 15  
 Thr Phe Arg Ile Leu Leu His Pro Glu Gln Pro Val Ala Phe Lys Ala  
 20 25 30  
 Gly Gln Tyr Leu Thr Val Val Met Gly Glu Lys Asp Lys Arg Pro Phe  
 35 40 45  
 Ser Ile Ala Ser Ser Pro Cys Arg His Glu Gly Glu Ile Glu Leu His  
 50 55 60  
 Ile Gly Ala Ala Glu His Asn Ala Tyr Ala Gly Glu Val Val Glu Ser  
 65 70 75 80  
 Met Lys Ser Ala Leu Glu Thr Gly Gly Asp Ile Leu Ile Asp Ala Pro  
 85 90 95  
 His Gly Glu Ala Trp Ile Arg Glu Asp Ser Asp Arg Ser Met Leu Leu  
 100 105 110  
 Ile Ala Gly Gly Thr Gly Phe Ser Tyr Val Arg Ser Ile Leu Asp His  
 115 120 125  
 Cys Ile Ser Gln Gln Ile Gln Lys Pro Ile Tyr Leu Tyr Trp Gly Gly  
 130 135 140  
 Arg Asp Glu Cys Gln Leu Tyr Ala Lys Ala Glu Leu Glu Ser Ile Ala  
 145 150 155 160  
 Gln Ala His Ser His Ile Thr Phe Val Pro Val Val Glu Lys Ser Glu  
 165 170 175  
 Gly Trp Thr Gly Lys Thr Gly Asn Val Leu Glu Ala Val Lys Ala Asp  
 180 185 190  
 Phe Asn Ser Leu Ala Asp Met Asp Ile Tyr Ile Ala Gly Arg Phe Glu  
 195 200 205

-25/43-

Met Ala Gly Ala Ala Arg Glu Gln Phe Thr Thr Glu Lys Gln Ala Lys  
 210 215 220

Lys Glu Gln Leu Phe Gly Asp Ala Phe Ala Phe Ile  
 225 230 235

## (2) INFORMATION FOR SEQ ID NO:15:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1079 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (iii) HYPOTHETICAL: NO

## (iv) ANTISENSE: NO

## (v) FRAGMENT TYPE:

## (vi) ORIGINAL SOURCE:

## (ix) FEATURE:

## (A) NAME/KEY: Coding Sequence

## (B) LOCATION: 259...975

(D) OTHER INFORMATION: *Renilla mulleri* GFP

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGTTATACAC	AAGTGTATCG	CGTATCTGCA	GACGCATCTA	GTGGGATTAT	TCGAGCGGTA	60
GTATTTACGT	CAGACCTGTC	TAATCGAAAC	CACAACAAAC	TCTTAAAATA	AGCCACATTT	120
ACATAATATC	TAAGAGACGC	CTCATTTAAG	AGTAGTAAAA	ATATAATATA	TGATAGAGTA	180
TACAACTCTC	GCCTTAGACA	GACAGTGTGC	AACAGAGTAA	CTCTTGTTAA	TGCAATCGAA	240
AGCGTCAAGA	GAGATAAG	ATG AGT AAA CAA ATA TTG	AAG AAC ACT TGT TTA			291
	Met Ser Lys Gln Ile Leu	Lys Asn Thr Cys Leu				
	1	5	10			
CAA GAA GTA ATG TCG TAT AAA GTA AAT CTG GAA GGA ATT GTA AAC AAC						339
Gln Glu Val Met Ser Tyr Lys Val Asn Leu Glu Gly Ile Val Asn Asn						
	15	20	25			
CAT GTT TTT ACA ATG GAG GGT TGC GGC AAA GGG AAT ATT TTA TTC GGC						387
His Val Phe Thr Met Glu Gly Cys Gly Lys Gly Asn Ile Leu Phe Gly						
	30	35	40			
AAT CAA CTG GTT CAG ATT CGT GTC ACG AAA GGG GCC CCA CTG CCT TTT						435
Asn Gln Leu Val Gln Ile Arg Val Thr Lys Gly Ala Pro Leu Pro Phe						
	45	50	55			
GCA TTT GAT ATT GTG TCA CCA GCT TTT CAA TAT GGC AAC CGT ACT TTC						483
Ala Phe Asp Ile Val Ser Pro Ala Phe Gln Tyr Gly Asn Arg Thr Phe						
	60	65	70	75		
ACG AAA TAT CCG AAT GAT ATA TCA GAT TAT TTT ATA CAA TCA TTT CCA						531
Thr Lys Tyr Pro Asn Asp Ile Ser Asp Tyr Phe Ile Gln Ser Phe Pro						
	80	85	90			
GCA GGA TTT ATG TAT GAA CGA ACA TTA CGT TAC GAA GAT GGC GGA CTT						579
Ala Gly Phe Met Tyr Glu Arg Thr Leu Arg Tyr Glu Asp Gly Gly Leu						
	95	100	105			
GTT GAA ATT CGT TCA GAT ATA AAT TTA ATA GAA GAC AAG TTC GTC TAC						627
Val Glu Ile Arg Ser Asp Ile Asn Leu Ile Glu Asp Lys Phe Val Tyr						

-26/43-

110	115	120	
AGA GTG GAA TAC AAA GGT AGT AAC TTC CCA GAT GAT GGT CCC GTC ATG			675
Arg Val Glu Tyr Lys Gly Ser Asn Phe Pro Asp Asp Gly Pro Val Met			
125	130	135	
CAG AAG ACT ATC TTA GGA ATA GAG CCT TCA TTT GAA GCC ATG TAC ATG			723
Gln Lys Thr Ile Leu Gly Ile Glu Pro Ser Phe Glu Ala Met Tyr Met			
140	145	150	155
AAT AAT GGC GTC TTG GTC GGC GAA GTA ATT CTT GTC TAT AAA CTA AAC			771
Asn Asn Gly Val Leu Val Gly Glu Val Ile Leu Val Tyr Lys Leu Asn			
	160	165	170
TCT GGG AAA TAT TAT TCA TGT CAC ATG AAA ACA TTA ATG AAG TCG AAA			819
Ser Gly Lys Tyr Tyr Ser Cys His Met Lys Thr Leu Met Lys Ser Lys			
	175	180	185
GGT GTA GTA AAG GAG TTT CCT TCG TAT CAT TTT ATT CAA CAT CGT TTG			867
Gly Val Val Lys Glu Phe Pro Ser Tyr His Phe Ile Gln His Arg Leu			
	190	195	200
GAA AAG ACT TAC GTA GAA GAC GGG GGG TTC GTT GAA CAG CAT GAG ACT			915
Glu Lys Thr Tyr Val Glu Asp Gly Gly Phe Val Glu Gln His Glu Thr			
	205	210	215
GCT ATT GCT CAA ATG ACA TCT ATA GGA AAA CCA CTA GGA TCC TTA CAC			963
Ala Ile Ala Gln Met Thr Ser Ile Gly Lys Pro Leu Gly Ser Leu His			
	220	225	230
GAA TGG GTT TAA ACACAGTTAC ATTACTTTTT CCAATTCGTG TTTCATGTCA AATAAT			1021
Glu Trp Val *			

AATTTTTTAA ACAATTATCA ATGTTTGTG ATATGTTTGT AAAAAAAAAA AAAAAAAAAA 1079

## (2) INFORMATION FOR SEQ ID NO:16:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 238 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (iii) HYPOTHETICAL: NO

## (iv) ANTISENSE: NO

## (v) FRAGMENT TYPE: internal

## (vi) ORIGINAL SOURCE:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Met	Ser	Lys	Gln	Ile	Leu	Lys	Asn	Thr	Cys	Leu	Gln	Glu	Val	Met	Ser
1				5					10					15	
Tyr	Lys	Val	Asn	Leu	Glu	Gly	Ile	Val	Asn	Asn	His	Val	Phe	Thr	Met
			20					25					30		
Glu	Gly	Cys	Gly	Lys	Gly	Asn	Ile	Leu	Phe	Gly	Asn	Gln	Leu	Val	Gln
		35				40					45				
Ile	Arg	Val	Thr	Lys	Gly	Ala	Pro	Leu	Pro	Phe	Ala	Phe	Asp	Ile	Val
	50					55				60					
Ser	Pro	Ala	Phe	Gln	Tyr	Gly	Asn	Arg	Thr	Phe	Thr	Lys	Tyr	Pro	Asn

-27/43-

65					70					75					80
Asp	Ile	Ser	Asp	Tyr	Phe	Ile	Gln	Ser	Phe	Pro	Ala	Gly	Phe	Met	Tyr
				85					90					95	
Glu	Arg	Thr	Leu	Arg	Tyr	Glu	Asp	Gly	Gly	Leu	Val	Glu	Ile	Arg	Ser
			100					105					110		
Asp	Ile	Asn	Leu	Ile	Glu	Asp	Lys	Phe	Val	Tyr	Arg	Val	Glu	Tyr	Lys
		115					120					125			
Gly	Ser	Asn	Phe	Pro	Asp	Asp	Gly	Pro	Val	Met	Gln	Lys	Thr	Ile	Leu
		130				135					140				
Gly	Ile	Glu	Pro	Ser	Phe	Glu	Ala	Met	Tyr	Met	Asn	Asn	Gly	Val	Leu
					150					155					160
Val	Gly	Glu	Val	Ile	Leu	Val	Tyr	Lys	Leu	Asn	Ser	Gly	Lys	Tyr	Tyr
				165					170					175	
Ser	Cys	His	Met	Lys	Thr	Leu	Met	Lys	Ser	Lys	Gly	Val	Val	Lys	Glu
			180					185					190		
Phe	Pro	Ser	Tyr	His	Phe	Ile	Gln	His	Arg	Leu	Glu	Lys	Thr	Tyr	Val
		195					200					205			
Glu	Asp	Gly	Gly	Phe	Val	Glu	Gln	His	Glu	Thr	Ala	Ile	Ala	Gln	Met
		210				215					220				
Thr	Ser	Ile	Gly	Lys	Pro	Leu	Gly	Ser	Leu	His	Glu	Trp	Val		
					230					235					

## (2) INFORMATION FOR SEQ ID NO:17:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1217 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: Genomic DNA

## (iii) HYPOTHETICAL: NO

## (iv) ANTISENSE: NO

## (v) FRAGMENT TYPE:

## (vi) ORIGINAL SOURCE:

## (ix) FEATURE:

## (A) NAME/KEY: Coding Sequence

## (B) LOCATION: 31...963

(D) OTHER INFORMATION: *Renilla mulleri* luciferase

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CGGCACGAGG	TTTAAGAATC	AATAAAAAAA	ATG	ACG	TCA	AAA	GTT	TAC	GAT	CCT	54					
			Met	Thr	Ser	Lys	Val	Tyr	Asp	Pro						
			1				5									
GAA	TTA	AGA	AAA	CGC	ATG	ATT	ACT	GGT	CCA	CAA	TGG	TGG	GCA	AGA	TGT	102
Glu	Leu	Arg	Lys	Arg	Met	Ile	Thr	Gly	Pro	Gln	Trp	Trp	Ala	Arg	Cys	
	10					15					20					
AAA	CAA	ATG	AAT	GTT	CTT	GAT	TCA	TTT	ATT	AAT	TAT	TAT	GAT	TCA	GAA	150
Lys	Gln	Met	Asn	Val	Leu	Asp	Ser	Phe	Ile	Asn	Tyr	Tyr	Asp	Ser	Glu	
	25				30					35					40	
AAA	CAT	GCA	GAA	AAT	GCA	GTT	ATA	TTT	TTA	CAT	GGT	AAT	GCA	GCA	TCT	198
Lys	His	Ala	Glu	Asn	Ala	Val	Ile	Phe	Leu	His	Gly	Asn	Ala	Ala	Ser	
				45					50					55		
TCT	TAT	TTA	TGG	CGT	CAT	GTT	GTA	CCA	CAT	GTT	GAA	CCA	GTG	GCG	CGA	246

-28/43-

Ser	Tyr	Leu	Trp	Arg	His	Val	Val	Pro	His	Val	Glu	Pro	Val	Ala	Arg	
			60					65					70			
TGT	ATT	ATA	CCA	GAT	CTT	ATA	GGT	ATG	GGT	AAA	TCA	GGC	AAG	TCT	GGT	294
Cys	Ile	Ile	Pro	Asp	Leu	Ile	Gly	Met	Gly	Lys	Ser	Gly	Lys	Ser	Gly	
		75					80					85				
AAT	GGT	TCC	TAT	AGA	TTA	CTA	GAT	CAT	TAC	AAA	TAT	CTT	ACT	GAA	TGG	342
Asn	Gly	Ser	Tyr	Arg	Leu	Leu	Asp	His	Tyr	Lys	Tyr	Leu	Thr	Glu	Trp	
	90					95					100					
TTC	AAA	CAT	CTT	AAT	TTA	CCA	AAG	AAG	ATC	ATT	TTT	GTC	GGT	CAT	GAT	390
Phe	Lys	His	Leu	Asn	Leu	Pro	Lys	Lys	Ile	Ile	Phe	Val	Gly	His	Asp	
105					110					115					120	
TGG	GGT	GCT	TGT	TTA	GCA	TTT	CAT	TAT	TGC	TAT	GAA	CAT	CAG	GAT	CGC	438
Trp	Gly	Ala	Cys	Leu	Ala	Phe	His	Tyr	Cys	Tyr	Glu	His	Gln	Asp	Arg	
				125					130					135		
ATC	AAA	GCA	GTT	GTT	CAT	GCT	GAA	AGT	GTA	GTA	GAT	GTG	ATT	GAA	TCG	486
Ile	Lys	Ala	Val	Val	His	Ala	Glu	Ser	Val	Val	Asp	Val	Ile	Glu	Ser	
			140					145					150			
TGG	GAC	GAA	TGG	CCT	GAT	ATT	GAA	GAA	GAT	ATT	GCT	TTG	ATT	AAA	TCT	534
Trp	Asp	Glu	Trp	Pro	Asp	Ile	Glu	Glu	Asp	Ile	Ala	Leu	Ile	Lys	Ser	
		155					160					165				
GAA	GAA	GGA	GAA	AAA	ATG	GTT	TTA	GAG	AAT	AAC	TTC	TTC	GTG	GAA	ACC	582
Glu	Glu	Gly	Glu	Lys	Met	Val	Leu	Glu	Asn	Asn	Phe	Phe	Val	Glu	Thr	
	170					175					180					
ATG	TTG	CCA	TCA	AAA	ATC	ATG	AGA	AAG	TTG	GAA	CCA	GAG	GAA	TTT	GCT	630
Met	Leu	Pro	Ser	Lys	Ile	Met	Arg	Lys	Leu	Glu	Pro	Glu	Glu	Phe	Ala	
185					190					195					200	
GCT	TAT	CTT	GAA	CCA	TTT	AAA	GAG	AAA	GGT	GAA	GTT	CGT	CGT	CCA	ACA	678
Ala	Tyr	Leu	Glu	Pro	Phe	Lys	Glu	Lys	Gly	Glu	Val	Arg	Arg	Pro	Thr	
				205					210					215		
TTA	TCA	TGG	CCT	CGT	GAA	ATC	CCT	TTG	GTA	AAA	GGT	GGT	AAA	CCG	GAT	726
Leu	Ser	Trp	Pro	Arg	Glu	Ile	Pro	Leu	Val	Lys	Gly	Gly	Lys	Pro	Asp	
			220				225						230			
GTA	GTA	GAA	ATT	GTC	AGG	AAT	TAT	AAT	GCT	TAT	CTT	CGT	GCA	AGT	CAT	774
Val	Val	Glu	Ile	Val	Arg	Asn	Tyr	Asn	Ala	Tyr	Leu	Arg	Ala	Ser	His	
		235					240					245				
GAT	TTA	CCA	AAA	ATG	TTT	ATT	GAA	TCT	GAT	CCA	GGA	TTC	TTT	TCC	AAT	822
Asp	Leu	Pro	Lys	Met	Phe	Ile	Glu	Ser	Asp	Pro	Gly	Phe	Phe	Ser	Asn	
	250					255					260					
GCT	ATT	GTT	GAA	GGT	GCT	AAG	AAA	TTC	CCT	AAT	ACT	GAA	TTT	GTT	AAA	870
Ala	Ile	Val	Glu	Gly	Ala	Lys	Lys	Phe	Pro	Asn	Thr	Glu	Phe	Val	Lys	
265					270				275						280	
GTC	AAA	GGT	CTT	CAT	TTT	TCA	CAA	GAA	GAT	GCA	CCT	GAT	GAA	ATG	GGA	918
Val	Lys	Gly	Leu	His	Phe	Ser	Gln	Glu	Asp	Ala	Pro	Asp	Glu	Met	Gly	
				285					290					295		
AAT	TAT	ATA	AAA	TCG	TTT	GTT	GAG	CGT	GTT	CTT	AAA	AAT	GAA	CAA	TAACT	969
Asn	Tyr	Ile	Lys	Ser	Phe	Val	Glu	Arg	Val	Leu	Lys	Asn	Glu	Gln		

-29/43-

300	305	310	
ACCAGGTTTC	CATGTTGCCA	CTTTAGCTGG	GTTTAATAAA
TTTCACATT	AATTTTAACT	ATTAAAAAAT	TATGGACACA
TTTAGTTGG	GAACAATGAA	TACCGAATAT	TATGAATTCT
CACATTCTTA	TGTAATAAAA	CTTTGTTTTA	ATAAATTAAT
AAAAAAA			
			1029
			1089
			1149
			1209
			1217

## (2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 311 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein  
 (iii) HYPOTHETICAL: NO  
 (iv) ANTISENSE: NO  
 (v) FRAGMENT TYPE: internal  
 (vi) ORIGINAL SOURCE:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Met	Thr	Ser	Lys	Val	Tyr	Asp	Pro	Glu	Leu	Arg	Lys	Arg	Met	Ile	Thr
1				5					10					15	
Gly	Pro	Gln	Trp	Trp	Ala	Arg	Cys	Lys	Gln	Met	Asn	Val	Leu	Asp	Ser
		20						25				30			
Phe	Ile	Asn	Tyr	Tyr	Asp	Ser	Glu	Lys	His	Ala	Glu	Asn	Ala	Val	Ile
		35					40				45				
Phe	Leu	His	Gly	Asn	Ala	Ala	Ser	Ser	Tyr	Leu	Trp	Arg	His	Val	Val
	50				55					60					
Pro	His	Val	Glu	Pro	Val	Ala	Arg	Cys	Ile	Ile	Pro	Asp	Leu	Ile	Gly
65					70				75					80	
Met	Gly	Lys	Ser	Gly	Lys	Ser	Gly	Asn	Gly	Ser	Tyr	Arg	Leu	Leu	Asp
			85					90					95		
His	Tyr	Lys	Tyr	Leu	Thr	Glu	Trp	Phe	Lys	His	Leu	Asn	Leu	Pro	Lys
		100						105					110		
Lys	Ile	Ile	Phe	Val	Gly	His	Asp	Trp	Gly	Ala	Cys	Leu	Ala	Phe	His
		115				120						125			
Tyr	Cys	Tyr	Glu	His	Gln	Asp	Arg	Ile	Lys	Ala	Val	Val	His	Ala	Glu
	130				135						140				
Ser	Val	Val	Asp	Val	Ile	Glu	Ser	Trp	Asp	Glu	Trp	Pro	Asp	Ile	Glu
145					150				155					160	
Glu	Asp	Ile	Ala	Leu	Ile	Lys	Ser	Glu	Glu	Gly	Glu	Lys	Met	Val	Leu
			165					170					175		
Glu	Asn	Asn	Phe	Val	Glu	Thr	Met	Leu	Pro	Ser	Lys	Ile	Met	Arg	
		180					185					190			
Lys	Leu	Glu	Pro	Glu	Glu	Phe	Ala	Ala	Tyr	Leu	Glu	Pro	Phe	Lys	Glu
		195					200					205			
Lys	Gly	Glu	Val	Arg	Arg	Pro	Thr	Leu	Ser	Trp	Pro	Arg	Glu	Ile	Pro
	210					215					220				
Leu	Val	Lys	Gly	Gly	Lys	Pro	Asp	Val	Val	Glu	Ile	Val	Arg	Asn	Tyr
225					230					235				240	
Asn	Ala	Tyr	Leu	Arg	Ala	Ser	His	Asp	Leu	Pro	Lys	Met	Phe	Ile	Glu
			245					250					255		
Ser	Asp	Pro	Gly	Phe	Phe	Ser	Asn	Ala	Ile	Val	Glu	Gly	Ala	Lys	Lys
		260						265					270		
Phe	Pro	Asn	Thr	Glu	Phe	Val	Lys	Val	Lys	Gly	Leu	His	Phe	Ser	Gln
		275					280					285			
Glu	Asp	Ala	Pro	Asp	Glu	Met	Gly	Asn	Tyr	Ile	Lys	Ser	Phe	Val	Glu

-30/43-

```

      290                      295                      300
Arg Val Leu Lys Asn Glu Gln
305                      310

```

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- ```

/ SEQUENCE CHARACTERISTICS
(A) LENGTH: 765 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

```

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence  
(B) LOCATION: 37...594  
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

|                  |                   |                   |                  |                  |                  |                   |                   |                  |                  |                  |                   |                   |                   |                  |            |     |
|------------------|-------------------|-------------------|------------------|------------------|------------------|-------------------|-------------------|------------------|------------------|------------------|-------------------|-------------------|-------------------|------------------|------------|-----|
| GCACGAGGGT       | ACTCAAAGTA        | TCTTCTGGCA        | GGGAAA           | ATG<br>Met<br>1  | GGA<br>Gly       | GTG<br>Val        | AAA<br>Lys        | GTT<br>Val<br>5  | CTT<br>Leu       |                  | 54                |                   |                   |                  |            |     |
| TTT<br>Phe       | GCC<br>Ala        | CTT<br>Leu        | ATT<br>Ile<br>10 | TGT<br>Cys       | ATT<br>Ile       | GCT<br>Ala        | GTG<br>Val        | GCC<br>Ala<br>15 | GAG<br>Glu       | GCC<br>Ala       | AAA<br>Lys        | CCA<br>Pro        | ACT<br>Thr<br>20  | GAA<br>Glu       | AAC<br>Asn | 102 |
| AAT<br>Asn       | GAA<br>Glu        | GAT<br>Asp<br>25  | TTC<br>Phe       | AAC<br>Asn       | ATT<br>Ile       | GTA<br>Val        | GCT<br>Ala<br>30  | GTA<br>Val       | GCT<br>Ala       | AGC<br>Ser       | AAC<br>Asn        | TTT<br>Phe<br>35  | GCT<br>Ala        | ACA<br>Thr       | ACG<br>Thr | 150 |
| GAT<br>Asp       | CTC<br>Leu<br>40  | GAT<br>Asp        | GCT<br>Ala       | GAC<br>Asp       | CGT<br>Arg       | GGT<br>Gly<br>45  | AAA<br>Lys        | TTG<br>Leu       | CCC<br>Pro       | GGA<br>Gly       | AAA<br>Lys<br>50  | AAA<br>Lys        | TTA<br>Leu        | CCA<br>Pro       | CTT<br>Leu | 198 |
| GAG<br>Glu<br>55 | GTA<br>Val        | CTC<br>Leu        | AAA<br>Lys       | GAA<br>Glu       | ATG<br>Met<br>60 | GAA<br>Glu        | GCC<br>Ala        | AAT<br>Asn       | GCT<br>Ala       | AGG<br>Arg<br>65 | AAA<br>Lys        | GCT<br>Ala        | GGC<br>Gly        | TGC<br>Cys<br>70 | ACT<br>Thr | 246 |
| AGG<br>Arg       | GGA<br>Gly        | TGT<br>Cys        | CTG<br>Leu       | ATA<br>Ile<br>75 | TGC<br>Cys       | CTG<br>Leu        | TCA<br>Ser        | CAC<br>His       | ATC<br>Ile<br>80 | AAG<br>Lys       | TGT<br>Cys        | ACA<br>Thr        | CCC<br>Pro        | AAA<br>Lys<br>85 | ATG<br>Met | 294 |
| AAG<br>Lys       | AAG<br>Lys        | TTT<br>Phe        | ATC<br>Ile<br>90 | CCA<br>Pro       | GGA<br>Gly       | AGA<br>Arg        | TGC<br>Cys        | CAC<br>His<br>95 | ACC<br>Thr       | TAT<br>Tyr       | GAA<br>Glu        | GGA<br>Gly        | GAC<br>Asp<br>100 | AAA<br>Lys       | GAA<br>Glu | 342 |
| AGT<br>Ser       | GCA<br>Ala        | CAG<br>Gln<br>105 | GGA<br>Gly       | GGA<br>Gly       | ATA<br>Ile       | GGA<br>Gly        | GAG<br>Glu<br>110 | GCT<br>Ala       | ATT<br>Ile       | GTT<br>Val       | GAC<br>Asp        | ATT<br>Ile<br>115 | CCT<br>Pro        | GAA<br>Glu       | ATT<br>Ile | 390 |
| CCT<br>Pro       | GGG<br>Gly<br>120 | TTT<br>Phe        | AAG<br>Lys       | GAT<br>Asp       | TTG<br>Leu       | GAA<br>Glu<br>125 | CCC<br>Pro        | ATG<br>Met       | GAA<br>Glu       | CAA<br>Gln       | TTC<br>Phe<br>130 | ATT<br>Ile        | GCA<br>Ala        | CAA<br>Gln       | GTT<br>Val | 438 |



-31/43-

|                                                                    |     |
|--------------------------------------------------------------------|-----|
| GAC CTA TGT GTA GAC TGC ACA ACT GGA TGC CTC AAA GGT CTT GCC AAT    | 486 |
| Asp Leu Cys Val Asp Cys Thr Thr Gly Cys Leu Lys Gly Leu Ala Asn    |     |
| 135 140 145 150                                                    |     |
| GTG CAA TGT TCT GAT TTA CTC AAG AAA TGG CTG CCA CAA AGA TGT GCA    | 534 |
| Val Gln Cys Ser Asp Leu Leu Lys Lys Trp Leu Pro Gln Arg Cys Ala    |     |
| 155 160 165                                                        |     |
| ACT TTT GCT AGC AAA ATT CAA GGC CAA GTG GAC AAA ATA AAG GGT GCC    | 582 |
| Thr Phe Ala Ser Lys Ile Gln Gly Gln Val Asp Lys Ile Lys Gly Ala    |     |
| 170 175 180                                                        |     |
| GGT GGT GAT TAA TCCTAATAGA ATACTGCATA ACTGGATGAT GATATACTAG CTTATT | 640 |
| Gly Gly Asp *                                                      |     |
| 185                                                                |     |
| GCTCATAAAA TGGCCATTTT TTGTAACAAA TCGAGTCTAT GTAATTCAAA ATACCTAATT  | 700 |
| AATTGTTAAT ACATATGTAA TTCCTATAAA TATAATTTAT GCAATCCAAA AAAAAAAAAA  | 760 |
| AAAAA                                                              | 765 |

## (2) INFORMATION FOR SEQ ID NO:20:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 186 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (iii) HYPOTHETICAL: NO

## (iv) ANTISENSE: NO

## (v) FRAGMENT TYPE: internal

## (vi) ORIGINAL SOURCE:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

|                                                                 |  |
|-----------------------------------------------------------------|--|
| Met Gly Val Lys Val Leu Phe Ala Leu Ile Cys Ile Ala Val Ala Glu |  |
| 1 5 10 15                                                       |  |
| Ala Lys Pro Thr Glu Asn Asn Glu Asp Phe Asn Ile Val Ala Val Ala |  |
| 20 25 30                                                        |  |
| Ser Asn Phe Ala Thr Thr Asp Leu Asp Ala Asp Arg Gly Lys Leu Pro |  |
| 35 40 45                                                        |  |
| Gly Lys Lys Leu Pro Leu Glu Val Leu Lys Glu Met Glu Ala Asn Ala |  |
| 50 55 60                                                        |  |
| Arg Lys Ala Gly Cys Thr Arg Gly Cys Leu Ile Cys Leu Ser His Ile |  |
| 65 70 75 80                                                     |  |
| Lys Cys Thr Pro Lys Met Lys Lys Phe Ile Pro Gly Arg Cys His Thr |  |
| 85 90 95                                                        |  |
| Tyr Glu Gly Asp Lys Glu Ser Ala Gln Gly Gly Ile Gly Glu Ala Ile |  |
| 100 105 110                                                     |  |
| Val Asp Ile Pro Glu Ile Pro Gly Phe Lys Asp Leu Glu Pro Met Glu |  |
| 115 120 125                                                     |  |
| Gln Phe Ile Ala Gln Val Asp Leu Cys Val Asp Cys Thr Thr Gly Cys |  |
| 130 135 140                                                     |  |
| Leu Lys Gly Leu Ala Asn Val Gln Cys Ser Asp Leu Leu Lys Lys Trp |  |
| 145 150 155 160                                                 |  |
| Leu Pro Gln Arg Cys Ala Thr Phe Ala Ser Lys Ile Gln Gly Gln Val |  |
| 165 170 175                                                     |  |
| Asp Lys Ile Lys Gly Ala Gly Gly Asp                             |  |
| 180 185                                                         |  |

-32/43-

## (2) INFORMATION FOR SEQ ID NO:21:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1146 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (iii) HYPOTHETICAL: NO

## (iv) ANTISENSE: NO

## (v) FRAGMENT TYPE:

## (vi) ORIGINAL SOURCE:

## (ix) FEATURE:

(A) NAME/KEY: CBD Coding Sequence

(B) LOCATION: 1...1146

(D) OTHER INFORMATION: DNA encoding a CBD-Gaussia luciferase fusion protein

## (ix) FEATURE:

(A) NAME/KEY: CBD Coding Sequence

(B) LOCATION: 1...591

## (ix) FEATURE:

(A) NAME/KEY: Gaussia luciferase

(B) LOCATION: 591-1146

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

|                                                                 |     |
|-----------------------------------------------------------------|-----|
| ATG TCA GTT GAA TTT TAC AAC TCT AAC AAA TCA GCA CAA ACA AAC TCA | 48  |
| Met Ser Val Glu Phe Tyr Asn Ser Asn Lys Ser Ala Gln Thr Asn Ser |     |
| 1 5 10 15                                                       |     |
| ATT ACA CCA ATA ATC AAA ATT ACT AAC ACA TCT GAC AGT GAT TTA AAT | 96  |
| Ile Thr Pro Ile Ile Lys Ile Thr Asn Thr Ser Asp Ser Asp Leu Asn |     |
| 20 25 30                                                        |     |
| TTA AAT GAC GTA AAA GTT AGA TAT TAT TAC ACA AGT GAT GGT ACA CAA | 144 |
| Leu Asn Asp Val Lys Val Arg Tyr Tyr Tyr Thr Ser Asp Gly Thr Gln |     |
| 35 40 45                                                        |     |
| GGA CAA ACT TTC TGG TGT GAC CAT GCT GGT GCA TTA TTA GGA AAT AGC | 192 |
| Gly Gln Thr Phe Trp Cys Asp His Ala Gly Ala Leu Leu Gly Asn Ser |     |
| 50 55 60                                                        |     |
| TAT GTT GAT AAC ACT AGC AAA GTG ACA GCA AAC TTC GTT AAA GAA ACA | 240 |
| Tyr Val Asp Asn Thr Ser Lys Val Thr Ala Asn Phe Val Lys Glu Thr |     |
| 65 70 75 80                                                     |     |
| GCA AGC CCA ACA TCA ACC TAT GAT ACA TAT GTT GAA TTT GGA TTT GCA | 288 |
| Ala Ser Pro Thr Ser Thr Tyr Asp Thr Tyr Val Glu Phe Gly Phe Ala |     |
| 85 90 95                                                        |     |
| AGC GGA GCA GCT ACT CTT AAA AAA GGA CAA TTT ATA ACT ATT CAA GGA | 336 |
| Ser Gly Ala Ala Thr Leu Lys Lys Gly Gln Phe Ile Thr Ile Gln Gly |     |
| 100 105 110                                                     |     |
| AGA ATA ACA AAA TCA GAC TGG TCA AAC TAC ACT CAA ACA AAT GAC TAT | 384 |
| Arg Ile Thr Lys Ser Asp Trp Ser Asn Tyr Thr Gln Thr Asn Asp Tyr |     |
| 115 120 125                                                     |     |
| TCA TTT GAT GCA AGT AGT TCA ACA CCA GTT GTA AAT CCA AAA GTT ACA | 432 |

-33/43-

|            |            |            |            |            |            |            |            |            |            |            |            |            |            |            |            |      |
|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------|
| Ser<br>130 | Phe        | Asp        | Ala        | Ser        | Ser        | Ser        | Thr        | Pro        | Val        | Val        | Asn<br>140 | Pro        | Lys        | Val        | Thr        |      |
| GGA<br>145 | TAT<br>Tyr | ATA<br>Ile | GGT<br>Gly | GGA<br>Gly | GCT<br>Ala | AAA<br>Lys | GTT<br>Val | CTT<br>Leu | GGT<br>Gly | ACA<br>Thr | GCA<br>Ala | CCA<br>Pro | GGT<br>Gly | TCC<br>Ser | GCG<br>Ala | 480  |
| GGT<br>Gly | CTG<br>Leu | GTG<br>Val | CCA<br>Pro | CGC<br>Arg | GGT<br>Gly | AGT<br>Ser | ACT<br>Thr | GCA<br>Ala | ATT<br>Ile | GGT<br>Gly | ATG<br>Met | AAA<br>Lys | GAA<br>Glu | ACC<br>Thr | GCT<br>Ala | 528  |
| GCT<br>Ala | GCT<br>Ala | AAA<br>Lys | TTC<br>Phe | GAA<br>Glu | CGC<br>Arg | CAG<br>Gln | CAC<br>His | ATG<br>Met | GAC<br>Asp | AGC<br>Ser | CCA<br>Pro | GAT<br>Asp | CTG<br>Leu | GGT<br>Gly | ACC<br>Thr | 576  |
| GAT<br>Asp | GAC<br>Asp | GAC<br>Asp | GAC<br>Asp | AAG<br>Lys | ATG<br>Met | GGA<br>Gly | GTG<br>Val | AAA<br>Lys | GTT<br>Val | CTT<br>Leu | TTT<br>Phe | GCC<br>Ala | CTT<br>Leu | ATT<br>Ile | TGT<br>Cys | 624  |
| ATT<br>Ile | GCT<br>Ala | GTG<br>Val | GCC<br>Ala | GAG<br>Glu | GCC<br>Ala | AAA<br>Lys | CCA<br>Pro | ACT<br>Thr | GAA<br>Glu | AAC<br>Asn | AAT<br>Asn | GAA<br>Glu | GAT<br>Asp | TTC<br>Phe | AAC<br>Asn | 672  |
| ATT<br>Ile | GTA<br>Val | GCT<br>Ala | GTA<br>Val | GCT<br>Ala | AGC<br>Ser | AAC<br>Asn | TTT<br>Phe | GCT<br>Ala | ACA<br>Thr | ACG<br>Thr | GAT<br>Asp | CTC<br>Leu | GAT<br>Asp | GCT<br>Ala | GAC<br>Asp | 720  |
| CGT<br>Arg | GGT<br>Gly | AAA<br>Lys | TTG<br>Leu | CCC<br>Pro | GGA<br>Gly | AAA<br>Lys | AAA<br>Lys | TTA<br>Leu | CCA<br>Pro | CTT<br>Leu | GAG<br>Glu | GTA<br>Val | CTC<br>Leu | AAA<br>Lys | GAA<br>Glu | 768  |
| ATG<br>Met | GAA<br>Glu | GCC<br>Ala | AAT<br>Asn | GCT<br>Ala | AGG<br>Arg | AAA<br>Lys | GCT<br>Ala | GGC<br>Gly | TGC<br>Cys | ACT<br>Thr | AGG<br>Arg | GGA<br>Gly | TGT<br>Cys | CTG<br>Leu | ATA<br>Ile | 816  |
| TGC<br>Cys | CTG<br>Leu | TCA<br>Ser | CAC<br>His | ATC<br>Ile | AAG<br>Lys | TGT<br>Cys | ACA<br>Thr | CCC<br>Pro | AAA<br>Lys | ATG<br>Met | AAG<br>Lys | AAG<br>Lys | TTT<br>Phe | ATC<br>Ile | CCA<br>Pro | 864  |
| GGA<br>Gly | AGA<br>Arg | TGC<br>Cys | CAC<br>His | ACC<br>Thr | TAT<br>Tyr | GAA<br>Glu | GGA<br>Gly | GAC<br>Asp | AAA<br>Lys | GAA<br>Glu | AGT<br>Ser | GCA<br>Ala | CAG<br>Gln | GGA<br>Gly | GGA<br>Gly | 912  |
| ATA<br>Ile | GGA<br>Gly | GAG<br>Glu | GCT<br>Ala | ATT<br>Ile | GTT<br>Val | GAC<br>Asp | ATT<br>Ile | CCT<br>Pro | GAA<br>Glu | ATT<br>Ile | CCT<br>Pro | GGG<br>Gly | TTT<br>Phe | AAG<br>Lys | GAT<br>Asp | 960  |
| TTG<br>Leu | GAA<br>Glu | CCC<br>Pro | ATG<br>Met | GAA<br>Glu | CAA<br>Gln | TTC<br>Phe | ATT<br>Ile | GCA<br>Ala | CAA<br>Gln | GTT<br>Val | GAC<br>Asp | CTA<br>Leu | TGT<br>Cys | GTA<br>Val | GAC<br>Asp | 1008 |
| TGC<br>Cys | ACA<br>Thr | ACT<br>Thr | GGA<br>Gly | TGC<br>Cys | CTC<br>Leu | AAA<br>Lys | GGT<br>Gly | CTT<br>Leu | GCC<br>Ala | AAT<br>Asn | GTG<br>Val | CAA<br>Gln | TGT<br>Cys | TCT<br>Ser | GAT<br>Asp | 1056 |
| TTA<br>Leu | CTC<br>Leu | AAG<br>Lys | AAA<br>Lys | TGG<br>Trp | CTG<br>Leu | CCA<br>Pro | CAA<br>Gln | AGA<br>Arg | TGT<br>Cys | GCA<br>Ala | ACT<br>Thr | TTT<br>Phe | GCT<br>Ala | AGC<br>Ser | AAA<br>Lys | 1104 |
| ATT<br>Ile | CAA<br>Gln | GGC<br>Gly | CAA<br>Gln | GTG<br>Val | GAC<br>Asp | AAA<br>Lys | ATA<br>Ile | AAG<br>Lys | GGT<br>Gly | GCC<br>Ala | GGT<br>Gly | GGT<br>Gly | GAT<br>Asp |            |            | 1146 |

-34/43-

370

375

380

## (2) INFORMATION FOR SEQ ID NO:22:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 382 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (iii) HYPOTHETICAL: NO

## (iv) ANTISENSE: NO

## (v) FRAGMENT TYPE: internal

## (vi) ORIGINAL SOURCE: CBD-Gaussia luciferase fusion protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```

Met Ser Val Glu Phe Tyr Asn Ser Asn Lys Ser Ala Gln Thr Asn Ser
 1      5      10      15
Ile Thr Pro Ile Ile Lys Ile Thr Asn Thr Ser Asp Ser Asp Leu Asn
 20      25      30
Leu Asn Asp Val Lys Val Arg Tyr Tyr Thr Ser Asp Gly Thr Gln
 35      40      45
Gly Gln Thr Phe Trp Cys Asp His Ala Gly Ala Leu Leu Gly Asn Ser
 50      55      60
Tyr Val Asp Asn Thr Ser Lys Val Thr Ala Asn Phe Val Lys Glu Thr
 65      70      75      80
Ala Ser Pro Thr Ser Thr Tyr Asp Thr Tyr Val Glu Phe Gly Phe Ala
 85      90      95
Ser Gly Ala Ala Thr Leu Lys Lys Gly Gln Phe Ile Thr Ile Gln Gly
100      105      110
Arg Ile Thr Lys Ser Asp Trp Ser Asn Tyr Thr Gln Thr Asn Asp Tyr
115      120      125
Ser Phe Asp Ala Ser Ser Ser Thr Pro Val Val Asn Pro Lys Val Thr
130      135      140
Gly Tyr Ile Gly Gly Ala Lys Val Leu Gly Thr Ala Pro Gly Ser Ala
145      150      155      160
Gly Leu Val Pro Arg Gly Ser Thr Ala Ile Gly Met Lys Glu Thr Ala
165      170      175      180
Ala Ala Lys Phe Glu Arg Gln His Met Asp Ser Pro Asp Leu Gly Thr
185      190      195
Asp Asp Asp Asp Lys Met Gly Val Lys Val Leu Phe Ala Leu Ile Cys
200      205
Ile Ala Val Ala Glu Ala Lys Pro Thr Glu Asn Asn Glu Asp Phe Asn
210      215      220
Ile Val Ala Val Ala Ser Asn Phe Ala Thr Thr Asp Leu Asp Ala Asp
225      230      235      240
Arg Gly Lys Leu Pro Gly Lys Lys Leu Pro Leu Glu Val Leu Lys Glu
245      250      255
Met Glu Ala Asn Ala Arg Lys Ala Gly Cys Thr Arg Gly Cys Leu Ile
260      265      270
Cys Leu Ser His Ile Lys Cys Thr Pro Lys Met Lys Lys Phe Ile Pro
275      280      285
Gly Arg Cys His Thr Tyr Glu Gly Asp Lys Glu Ser Ala Gln Gly Gly
290      295      300
Ile Gly Glu Ala Ile Val Asp Ile Pro Glu Ile Pro Gly Phe Lys Asp
305      310      315      320
Leu Glu Pro Met Glu Gln Phe Ile Ala Gln Val Asp Leu Cys Val Asp
325      330      335

```

-35/43-

Cys Thr Thr Gly Cys Leu Lys Gly Leu Ala Asn Val Gln Cys Ser Asp  
                   340                  345                  350  
 Leu Leu Lys Lys Trp Leu Pro Gln Arg Cys Ala Thr Phe Ala Ser Lys  
                   355                  360                  365  
 Ile Gln Gly Gln Val Asp Lys Ile Lys Gly Ala Gly Gly Asp  
       370                  375                  380

## (2) INFORMATION FOR SEQ ID NO:23

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (iii) HYPOTHETICAL: NO

## (iv) ANTISENSE: NO

## (v) FRAGMENT TYPE: N-terminal

## (vi) ORIGINAL SOURCE:

## (A) NAME/KEY:

## (B) LOCATION:

(D) OTHER INFORMATION: Amino-terminal *Renilla reniformis* GFP peptide (Identity of residue #5 unknown)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Met Asp Leu Ala Xaa Lys Leu Gly Leu Lys Glu Val Met Pro Thr Lys  
   1                  5                  10                  15  
 Ile Asn Leu Glu Gly Leu Val Gly Asp His Ala Phe Ser Met Glu Gly  
                   20                  25                  30  
 Val Gly Glu Gly Asn Ile Leu Glu Gly  
       35                  40

## (2) INFORMATION FOR SEQ ID NO:24:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (iii) HYPOTHETICAL: NO

## (iv) ANTISENSE: NO

## (v) FRAGMENT TYPE: N-terminal

## (vi) ORIGINAL SOURCE:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Val Lys Ile Ser Val Thr Lys Gly Ala Pro Leu Pro Phe Ala Phe Asp Ile  
   1                  5                  10                  15  
 Val Ser Val Ala Phe  
       20

## (2) INFORMATION FOR SEQ ID NO: 25:

## (i) SEQUENCE CHARACTERISTICS:

-36/43-

- (A) LENGTH: 40 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE: N-terminal
- (vi) ORIGINAL SOURCE:

- (A) NAME/KEY:
- (B) LOCATION:
- (D) OTHER INFORMATION: *Renilla reniformis* GFP peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Ala Tyr Thr Gly Tyr Pro Glu Glu Ile Ser Asp Tyr Phe Leu Gln Ser Phe  
1 5 10 15  
Pro Glu Gly Phe Thr Tyr Glu Arg Gly Asn Ile Arg Tyr Gln Asp Gly Gly  
20 25 30  
Thr Ala Ile Val Lys Ser  
35 40

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 12 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE: N-terminal
- (vi) ORIGINAL SOURCE:

- (A) NAME/KEY:
- (B) LOCATION:
- (D) OTHER INFORMATION: *Renilla reniformis* GFP peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Ile Val Gly Met Gln Pro Ser Tyr Glu Ser Met Tyr  
1 5 10

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 81 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE: N-terminal

-37/43-

## (vi) ORIGINAL SOURCE:

(A) NAME/KEY:  
 (B) LOCATION:  
 (D) OTHER INFORMATION: Carboxyl-terminal *Renilla reniformis* GFP  
 peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Thr Asn Val Thr Ser Val Ile Gly Gln Ile Ile Ala Phe Lys Leu Gln  
 1 5 10 15  
 Thr Gly Lys His Phe Thr Tyr His Met Arg Thr Val Tyr Lys Ser Lys  
 20 25 30  
 Lys Pro Val Glu Thr Met Pro Leu Tyr His Phe Ile Gln His Arg Leu  
 35 40 45  
 Val Lys Thr Asn Val Asp Thr Ala Ser Gly Tyr Val Val Gln His Glu  
 50 55 60  
 Thr Ala Ile Ala Ala His Ser Thr Ile Lys Lys Ile Glu Gly Ala Ala  
 65 70 75 80  
 Arg

## (2) INFORMATION FOR SEQ ID NO:28:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 861 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: cDNA

## (vi) ORIGINAL SOURCE: Pleuromamma

## (ix) FEATURE:

(A) NAME/KEY: Coding Sequence  
 (B) LOCATION: 148...741  
 (D) OTHER INFORMATION: Pleuromamma luciferase

## (x) PUBLICATION INFORMATION:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CGGCACGAGA TTTTGTCTGT GGTGATTGGG ATTGTCTGTC TCTCAGGTCA AGCAGAAAGT 60  
 TCGCTGAAAG GTGATTCTCTG TAGTGATGTT TCCTTCTGGG ATGTGATCAA GTACAACACT 120  
 GAGAGTCGAC AATGCTGTGA CACAAAA ATG CTT AGA AAT TGC GCT AGG AAG CAA 174  
 Met Leu Arg Asn Cys Ala Arg Lys Gln  
 1 5  
 GAG CAA GTT TGC GCC GAT GTG ACC GAG ATG AAA TGC CAA GCA GTT GCT 222  
 Glu Gln Val Cys Ala Asp Val Thr Glu Met Lys Cys Gln Ala Val Ala  
 10 15 20 25  
 TGG GCC GAC TGT GGA CCC AGA TTT GAT TCC ACT GGC AGG AAT AGA TGC 270

-38/43-

|            |            |            |            |            |            |     |     |     |     |     |     |     |     |     |     |     |  |
|------------|------------|------------|------------|------------|------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|
| Trp        | Ala        | Asp        | Cys        | Gly        | Pro        | Arg | Phe | Asp | Ser | Thr | Gly | Arg | Asn | Arg | Cys |     |  |
|            |            |            |            | 30         |            |     |     |     | 35  |     |     |     |     | 40  |     |     |  |
| CAA        | GTT        | CAA        | TAC        | AAG        | GAC        | TAC | GCG | TAC | AAG | TCC | TGC | GTG | GAA | GTT | GAT | 318 |  |
| Gln        | Val        | Gln        | Tyr        | Lys        | Asp        | Tyr | Ala | Tyr | Lys | Ser | Cys | Val | Glu | Val | Asp |     |  |
|            |            |            | 45         |            |            |     |     | 50  |     |     |     |     | 55  |     |     |     |  |
| TAC        | ACT        | GTA        | CCG        | CAC        | AGG        | AAG | CAA | GTT | CCA | GAG | TGC | AAA | CAA | GTC | ACT | 366 |  |
| Tyr        | Thr        | Val        | Pro        | His        | Arg        | Lys | Gln | Val | Pro | Glu | Cys | Lys | Gln | Val | Thr |     |  |
|            |            | 60         |            |            |            |     | 65  |     |     |     |     | 70  |     |     |     |     |  |
| AAA        | GAT        | AAC        | TGC        | GTT        | ACT        | GAT | TGG | GAA | GTT | GAC | GCC | AAT | GGC | AAC | AAG | 414 |  |
| Lys        | Asp        | Asn        | Cys        | Val        | Thr        | Asp | Trp | Glu | Val | Asp | Ala | Asn | Gly | Asn | Lys |     |  |
|            | 75         |            |            |            |            | 80  |     |     |     |     | 85  |     |     |     |     |     |  |
| GTT        | TGG        | GGT        | GGT        | ACC        | GAG        | AAA | TGC | ACT | CCT | GTC | ACT | TGG | GAA | GAA | TGT | 462 |  |
| Val        | Trp        | Gly        | Gly        | Thr        | Glu        | Lys | Cys | Thr | Pro | Val | Thr | Trp | Glu | Glu | Cys |     |  |
|            | 90         |            |            |            | 95         |     |     |     | 100 |     |     |     |     |     | 105 |     |  |
| AAT        | ATC        | GTG        | GAG        | AAA        | GAT        | GTA | GAT | TTT | CCA | ACT | GTC | AAG | ACG | GAA | TGC | 510 |  |
| Asn        | Ile        | Val        | Glu        | Lys        | Asp        | Val | Asp | Phe | Pro | Thr | Val | Lys | Thr | Glu | Cys |     |  |
|            |            |            |            |            | 110        |     |     |     | 115 |     |     |     |     |     | 120 |     |  |
| GGC        | ATC        | CTG        | TCT        | CAC        | CTT        | AAG | TAT | GCA | GAC | TTC | ATA | GAG | GGA | CCT | TCC | 558 |  |
| Gly        | Ile        | Leu        | Ser        | His        | Leu        | Lys | Tyr | Ala | Asp | Phe | Ile | Glu | Gly | Pro | Ser |     |  |
|            |            |            | 125        |            |            |     |     | 130 |     |     |     |     | 135 |     |     |     |  |
| CAC        | TCT        | TTG        | TCT        | ATG        | AGA        | ACC | AAT | TGT | CAG | GTC | AAG | AGT | TCA | TTG | GAC | 606 |  |
| His        | Ser        | Leu        | Ser        | Met        | Arg        | Thr | Asn | Cys | Gln | Val | Lys | Ser | Ser | Leu | Asp |     |  |
|            |            |            | 140        |            |            |     |     | 145 |     |     |     |     | 150 |     |     |     |  |
| TGC        | CGG        | CCT        | GTT        | AAG        | ACC        | AGG | AAG | TGT | GCA | ACG | GTC | GAG | TAC | CAC | GAA | 654 |  |
| Cys        | Arg        | Pro        | Val        | Lys        | Thr        | Arg | Lys | Cys | Ala | Thr | Val | Glu | Tyr | His | Glu |     |  |
|            |            | 155        |            |            |            |     | 160 |     |     |     |     | 165 |     |     |     |     |  |
| TGC        | AGC        | ATG        | AAG        | CCC        | CAA        | GAA | GAC | TGC | AGC | CCA | GTC | ACT | GTT | CAT | ATT | 702 |  |
| Cys        | Ser        | Met        | Lys        | Pro        | Gln        | Glu | Asp | Cys | Ser | Pro | Val | Thr | Val | His | Ile |     |  |
|            | 170        |            |            |            | 175        |     |     |     | 180 |     |     |     |     |     | 185 |     |  |
| CCT        | GAC        | CAG        | GAG        | AAA        | GTT        | CAC | CAG | AAG | AAG | TGC | CTC | ACA | TAA |     |     | 744 |  |
| Pro        | Asp        | Gln        | Glu        | Lys        | Val        | His | Gln | Lys | Lys | Cys | Leu | Thr |     |     |     |     |  |
|            |            |            |            |            | 190        |     |     |     | 195 |     |     |     |     |     |     |     |  |
| ATGTTATCAA | TTTAGCTCT  | TACTAATTTA | AACATAATAA | ATATCACATC | GAAGCCCTTT | 804 |     |     |     |     |     |     |     |     |     |     |  |
| ATTTTATAGA | AGTGTAATGC | TTGAATAAAT | CTAGTGAATA | AAAAAAAAAA | AAAAAAA    | 861 |     |     |     |     |     |     |     |     |     |     |  |

## (2) INFORMATION FOR SEQ ID NO:29:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 198 amino acids
- (B) TYPE: polypeptide
- (C) STRANDEDNESS: N/A
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: protein

- (A) NAME/KEY: Pleuromamma luciferase
- (B) LOCATION:
- (D) OTHER INFORMATION: Pleuromamma luciferase



-39/43-

(vi) ORIGINAL SOURCE: Pleuromamma

(x) PUBLICATION INFORMATION

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

```

Met Leu Arg Asn Cys Ala Arg Lys Gln Glu Gln Val Cys Ala Asp Val
 1           5          10          15
Thr Glu Met Lys Cys Gln Ala Val Ala Trp Ala Asp Cys Gly Pro Arg
          20          25          30
Phe Asp Ser Thr Gly Arg Asn Arg Cys Gln Val Gln Tyr Lys Asp Thr
          35          40          45
Ala Tyr Lys Ser Cys Val Glu Val Asp Tyr Thr Val Pro His Arg Lys
          50          55          60
Gln Val Pro Glu Cys Lys Gln Val Thr Lys Asp Asn Cys Val Thr Asp
65          70          75          80
Trp Glu Val Asp Ala Asn Gly Asn Lys Val Trp Gly Gly Thr Glu Lys
          85          90          95
Cys Thr Pro Val Thr Trp Glu Glu Cys Asn Ile Val Glu Lys Asp Val
          100          105          110
Asp Phe Pro Thr Val Lys Thr Glu Cys Gly Ile Leu Ser His Leu Lys
          115          120          125
Tyr Ala Asp Phe Ile Glu Gly Pro Ser His Ser Leu Ser Met Arg Thr
          130          135          140
Asn Cys Gln Val Lys Ser Ser Leu Asp Cys Arg Pro Val Lys Thr Arg
145          150          155          160
Lys Cys Ala Thr Val Glu Tyr His Glu Cys Ser Met Lys Pro Gln Glu
          165          170          175
Asp Cys Ser Pro Val Thr Val His Ile Pro Asp Gln Glu Lys Val His
          180          185          190
Gln Lys Lys Cys Leu Thr
          195

```

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1104 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE: Ptilosarcus gurneyi

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence (Insert A)

-40/43-

(B) LOCATION: 34...747

(D) OTHER INFORMATION: Ptilosarcus Green Fluorescent Protein

(GFP)

(x) PUBLICATION INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

|       |        |       |        |            |     |     |     |     |     |     |     |     |     |     |     |     |
|-------|--------|-------|--------|------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| TCGGC | CACGAG | CTGGC | CCTCCA | CACTTTAGAC | AAA | ATG | AAC | CGC | AAC | GTA | TTA | AAG | 54  |     |     |     |
|       |        |       |        |            |     | Met | Asn | Arg | Asn | Val | Leu | Lys |     |     |     |     |
|       |        |       |        |            |     | 1   |     |     |     | 5   |     |     |     |     |     |     |
| AAC   | ACT    | GGA   | CTG    | AAA        | GAG | ATT | ATG | TCG | GCA | AAA | GCT | AGC | GTT | GAA | GGA | 102 |
| Asn   | Thr    | Gly   | Leu    | Lys        | Glu | Ile | Met | Ser | Ala | Lys | Ala | Ser | Val | Glu | Gly |     |
|       |        | 10    |        |            |     | 15  |     |     |     |     | 20  |     |     |     |     |     |
| ATC   | GTG    | AAC   | AAT    | CAC        | GTT | TTT | TCC | ATG | GAA | GGA | TTT | GGA | AAA | GGC | AAT | 150 |
| Ile   | Val    | Asn   | Asn    | His        | Val | Phe | Ser | Met | Glu | Gly | Phe | Gly | Lys | Gly | Asn |     |
|       | 25     |       |        |            | 30  |     |     |     |     | 35  |     |     |     |     |     |     |
| GTA   | TTA    | TTT   | GGA    | AAC        | CAA | TTG | ATG | CAA | ATC | CGG | GTT | ACA | AAG | GGA | GGT | 198 |
| Val   | Leu    | Phe   | Gly    | Asn        | Gln | Leu | Met | Gln | Ile | Arg | Val | Thr | Lys | Gly | Gly |     |
| 40    |        |       |        | 45         |     |     |     | 50  |     |     |     |     |     | 55  |     |     |
| CCG   | TTG    | CCA   | TTC    | GCT        | TTC | GAT | ATT | GTT | TCC | ATA | GCT | TTC | CAA | TAC | GGG | 246 |
| Pro   | Leu    | Pro   | Phe    | Ala        | Phe | Asp | Ile | Val | Ser | Ile | Ala | Phe | Gln | Tyr | Gly |     |
|       |        |       | 60     |            |     | 65  |     |     |     |     |     |     | 70  |     |     |     |
| AAT   | CGC    | ACT   | TTC    | ACG        | AAA | TAC | CCA | GAC | GAC | ATT | GCG | GAC | TAC | TTT | GTT | 294 |
| Asn   | Arg    | Thr   | Phe    | Thr        | Lys | Tyr | Pro | Asp | Asp | Ile | Ala | Asp | Tyr | Phe | Val |     |
|       |        | 75    |        |            |     | 80  |     |     |     |     |     | 85  |     |     |     |     |
| CAA   | TCA    | TTC   | CCG    | GCT        | GGA | TTT | TTC | TAC | GAA | AGA | AAT | CTA | CGC | TTT | GAA | 342 |
| Gln   | Ser    | Phe   | Pro    | Ala        | Gly | Phe | Phe | Tyr | Glu | Arg | Asn | Leu | Arg | Phe | Glu |     |
|       | 90     |       |        |            | 95  |     |     |     |     |     | 100 |     |     |     |     |     |
| GAT   | GGC    | GCC   | ATT    | GTT        | GAC | ATT | CGT | TCA | GAT | ATA | AGT | TTA | GAA | GAT | GAT | 390 |
| Asp   | Gly    | Ala   | Ile    | Val        | Asp | Ile | Arg | Ser | Asp | Ile | Ser | Leu | Glu | Asp | Asp |     |
|       | 105    |       |        |            | 110 |     |     |     |     | 115 |     |     |     |     |     |     |
| AAG   | TTC    | CAC   | TAC    | AAA        | GTG | GAG | TAT | AGA | GGC | AAC | GGT | TTC | CCT | AGT | AAC | 438 |
| Lys   | Phe    | His   | Tyr    | Lys        | Val | Glu | Tyr | Arg | Gly | Asn | Gly | Phe | Pro | Ser | Asn |     |
| 120   |        |       |        | 125        |     |     |     |     | 130 |     |     |     |     |     | 135 |     |
| GGA   | CCC    | GTG   | ATG    | CAA        | AAA | GCC | ATC | CTC | GGC | ATG | GAG | CCA | TCG | TTT | GAG | 486 |
| Gly   | Pro    | Val   | Met    | Gln        | Lys | Ala | Ile | Leu | Gly | Met | Glu | Pro | Ser | Phe | Glu |     |
|       |        |       | 140    |            |     |     |     | 145 |     |     |     |     | 150 |     |     |     |
| GTG   | GTC    | TAC   | ATG    | AAC        | AGC | GGC | GTT | CTG | GTG | GGC | GAA | GTA | GAT | CTC | GTT | 534 |
| Val   | Val    | Tyr   | Met    | Asn        | Ser | Gly | Val | Leu | Val | Gly | Glu | Val | Asp | Leu | Val |     |
|       |        | 155   |        |            |     | 160 |     |     |     |     |     | 165 |     |     |     |     |
| TAC   | AAA    | CTC   | GAG    | TCA        | GGG | AAC | TAT | TAC | TCG | TGC | CAC | ATG | AAA | ACG | TTT | 582 |
| Tyr   | Lys    | Leu   | Glu    | Ser        | Gly | Asn | Tyr | Tyr | Ser | Cys | His | Met | Lys | Thr | Phe |     |
|       | 170    |       |        |            | 175 |     |     |     |     |     | 180 |     |     |     |     |     |
| TAC   | AGA    | TCC   | AAA    | GGT        | GGA | GTG | AAA | GAA | TTC | CCG | GAA | TAT | CAC | TTT | ATC | 630 |
| Tyr   | Arg    | Ser   | Lys    | Gly        | Gly | Val | Lys | Glu | Phe | Pro | Glu | Tyr | His | Phe | Ile |     |
|       | 185    |       |        |            | 190 |     |     |     |     |     | 195 |     |     |     |     |     |

-41/43-

```

CAT CAT CGT CTG GAG AAA ACC TAC GTG GAA GAA GGA AGC TTC GTG GAA 678
His His Arg Leu Glu Lys Thr Tyr Val Glu Glu Gly Ser Phe Val Glu
200                205                210                215

CAA CAC GAG ACG GCC ATT GCA CAA CTG ACC ACA ATT GGA AAA CCT CTG 726
Gln His Glu Thr Ala Ile Ala Gln Leu Thr Thr Ile Gly Lys Pro Leu
                220                225                230

GGC TCC CTT CAT GAA TGG GTG TAG AAAATGACCA ATATACTGGG GAAACCGATA 780
Gly Ser Leu His Glu Trp Val
                235

ACCGTTTGGG AGCTTGTGTA TACAAATTAT TTGGGGTTCAT TTTGTAATGT GTATGTGTGT 840
TGTATGATCA ATAGACGTCG TCATTCATAG CTTGAATCCT TCAGCAAAAG AAACCTCGAA 900
GCATATTGAA ACCTCGAAGC ATATTGAAAC CTCGACGGAG AGCGTAAAGA GACCGCACAA 960
ATTAACGCGT TTCAACCAGC AGTTGGAATC TTTAAACCGA TCAAAACTAT TAATATAAAT 1020
ATATATACCC TGTATAACTT ATATATATCT ATATAGTTTG ATATTGATTA AATCTGTTCT 1080
TGATCAAAAA AAAAAAAAAA AAAA 1104

```

## (2) INFORMATION FOR SEQ ID NO:31:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1279 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE: *Ptilosarcus gurneyi*

## (ix) FEATURE:

- (A) NAME/KEY: Coding Sequence (Insert B)
- (B) LOCATION: 7...720
- (D) OTHER INFORMATION: *Ptilosarcus* Green Fluorescent Protein

(GFP)

## (x) PUBLICATION INFORMATION:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

```

GACAAA ATG AAC CGC AAC GTA TTA AAG 27
Met Asn Arg Asn Val Leu Lys
1 5

AAC ACT GGA CTG AAA GAG ATT ATG TCG GCA AAA GCT AGC GTT GAA GGA 75
Asn Thr Gly Leu Lys Glu Ile Met Ser Ala Lys Ala Ser Val Glu Gly
10 15 20

ATC GTG AAC AAT CAC GTT TTT TCC ATG GAA GGA TTT GGA AAA GGC AAT 123
Ile Val Asn Asn His Val Phe Ser Met Glu Gly Phe Gly Lys Gly Asn
25 30 35

GTA TTA TTT GGA AAC CAA TTG ATG CAA ATC CGG GTT ACA AAG GGA GGT 171
Val Leu Phe Gly Asn Gln Leu Met Gln Ile Arg Val Thr Lys Gly Gly
40 45 50 55

CCG TTG CCA TTC GCT TTC GAC ATT GTT TCC ATA GCT TTC CAA TAC GGG 219

```

-42/43-

|            |             |            |              |             |             |     |     |            |            |            |     |     |     |     |     |      |
|------------|-------------|------------|--------------|-------------|-------------|-----|-----|------------|------------|------------|-----|-----|-----|-----|-----|------|
| Pro        | Leu         | Pro        | Phe          | Ala         | Phe         | Asp | Ile | Val        | Ser        | Ile        | Ala | Phe | Gln | Tyr | Gly |      |
|            |             |            |              | 60          |             |     |     |            | 65         |            |     |     |     | 70  |     |      |
| AAT        | CGC         | ACT        | TTC          | ACG         | AAA         | TAC | CCA | GAC        | GAC        | ATT        | GCG | GAC | TAC | TTT | GTT | 267  |
| Asn        | Arg         | Thr        | Phe          | Thr         | Lys         | Tyr | Pro | Asp        | Asp        | Ile        | Ala | Asp | Tyr | Phe | Val |      |
|            |             |            | 75           |             |             |     |     | 80         |            |            |     |     | 85  |     |     |      |
| CAA        | TCA         | TTT        | CCG          | GCT         | GGA         | TTT | TTC | TAC        | GAA        | AGA        | AAT | CTA | CGC | TTT | GAA | 315  |
| Gln        | Ser         | Phe        | Pro          | Ala         | Gly         | Phe | Phe | Tyr        | Glu        | Arg        | Asn | Leu | Arg | Phe | Glu |      |
|            |             |            | 90           |             |             |     | 95  |            |            |            |     | 100 |     |     |     |      |
| GAT        | GGC         | GCC        | ATT          | GTT         | GAC         | ATT | CGT | TCA        | GAT        | ATA        | AGT | TTA | GAA | GAT | GAT | 363  |
| Asp        | Gly         | Ala        | Ile          | Val         | Asp         | Ile | Arg | Ser        | Asp        | Ile        | Ser | Leu | Glu | Asp | Asp |      |
|            | 105         |            |              |             |             | 110 |     |            |            |            | 115 |     |     |     |     |      |
| AAG        | TTC         | CAC        | TAC          | AAA         | GTG         | GAG | TAT | AGA        | GGC        | AAC        | GGT | TTC | CCT | AGT | AAC | 411  |
| Lys        | Phe         | His        | Tyr          | Lys         | Val         | Glu | Tyr | Arg        | Gly        | Asn        | Gly | Phe | Pro | Ser | Asn |      |
|            | 120         |            |              |             | 125         |     |     |            |            | 130        |     |     |     |     | 135 |      |
| GGA        | CCC         | GTG        | ATG          | CAA         | AAA         | GCC | ATC | CTC        | GGC        | ATG        | GAG | CCA | TCG | TTT | GAG | 459  |
| Gly        | Pro         | Val        | Met          | Gln         | Lys         | Ala | Ile | Leu        | Gly        | Met        | Glu | Pro | Ser | Phe | Glu |      |
|            |             |            |              | 140         |             |     |     |            | 145        |            |     |     |     | 150 |     |      |
| GTG        | GTC         | TAC        | ATG          | AAC         | AGC         | GGC | GTT | CTG        | GTG        | GGC        | GAA | GTA | GAT | CTC | GTT | 507  |
| Val        | Val         | Tyr        | Met          | Asn         | Ser         | Gly | Val | Leu        | Val        | Gly        | Glu | Val | Asp | Leu | Val |      |
|            |             |            | 155          |             |             |     |     | 160        |            |            |     |     | 165 |     |     |      |
| TAC        | AAA         | CTC        | GAG          | TCA         | GGG         | AAC | TAT | TAC        | TCG        | TGC        | CAC | ATG | AAA | ACG | TTT | 555  |
| Tyr        | Lys         | Leu        | Glu          | Ser         | Gly         | Asn | Tyr | Tyr        | Ser        | Cys        | His | Met | Lys | Thr | Phe |      |
|            |             |            | 170          |             |             |     | 175 |            |            |            |     | 180 |     |     |     |      |
| TAC        | AGA         | TCC        | AAA          | GGT         | GGA         | GTG | AAA | GAA        | TTC        | CCG        | GAA | TAT | CAC | TTT | ATC | 603  |
| Tyr        | Arg         | Ser        | Lys          | Gly         | Gly         | Val | Lys | Glu        | Phe        | Pro        | Glu | Tyr | His | Phe | Ile |      |
|            |             |            | 185          |             |             | 190 |     |            |            |            | 195 |     |     |     |     |      |
| CAT        | CAT         | CGT        | CTG          | GAG         | AAA         | ACC | TAC | GTG        | GAA        | GAA        | GGA | AGC | TTC | GTG | GAA | 651  |
| His        | His         | Arg        | Leu          | Glu         | Lys         | Thr | Tyr | Val        | Glu        | Glu        | Gly | Ser | Phe | Val | Glu |      |
|            |             |            |              |             | 205         |     |     |            |            | 210        |     |     |     |     | 215 |      |
| CAA        | CAC         | GAG        | ACG          | GCC         | ATT         | GCA | CAA | CTG        | ACC        | ACA        | ATT | GGA | AAA | CCT | CTG | 699  |
| Gln        | His         | Glu        | Thr          | Ala         | Ile         | Ala | Gln | Leu        | Thr        | Thr        | Ile | Gly | Lys | Pro | Leu |      |
|            |             |            |              | 220         |             |     |     | 225        |            |            |     |     |     | 230 |     |      |
| GGC        | TCC         | CTT        | CAT          | GAA         | TGG         | GTG | TAG | AAAATGACCA | ATATACTGGG | GAAAATCACC |     |     |     |     |     | 753  |
| Gly        | Ser         | Leu        | His          | Glu         | Trp         | Val |     |            |            |            |     |     |     |     |     |      |
| AATATACTGG | GGAAAATGAC  | CAATTTACTG | GGGAAAATGA   | CCAATATACT  | GTAGAAAATC  |     |     |            |            |            |     |     |     |     |     | 813  |
| ACCAATATAC | TGGGGGAAAAT | GACCAATTTA | CTGGGGGAAAAT | GACCAATTTA  | CTGTAGAAAA  |     |     |            |            |            |     |     |     |     |     | 873  |
| TCACCAATAT | ACTGTGGAAA  | ATGACCAAAA | TACTGTAGAA   | ATGTTTCACAC | TGGGTTGATA  |     |     |            |            |            |     |     |     |     |     | 933  |
| ACCGTTTCGA | TAACCGTTTG  | GAAGCTTGTG | TATACAAGTT   | ATTTGGGGTC  | ATTTTGTAAAT |     |     |            |            |            |     |     |     |     |     | 993  |
| GTGTATGTGT | GTGTATGAT   | CTATAGACGT | CGTCATTTCAT  | AGCTTGAATC  | CTTCAGCAAA  |     |     |            |            |            |     |     |     |     |     | 1053 |
| AGAAACCTCG | AAGCATATTG  | AAACCTCGAC | GGAGAGCATA   | AAGAGACCGC  | ACGTACACAA  |     |     |            |            |            |     |     |     |     |     | 1113 |
| ATTATAATAC | CAGCAGTTGG  | AATCTTTAAA | CCGATCAAAA   | CTATTAATAT  | ATATATACAC  |     |     |            |            |            |     |     |     |     |     | 1173 |
| CCTGTATAAC | ATATATATAT  | ATATATATCT | ACATAGTTTG   | ATATTGATTA  | AATCTGTTCT  |     |     |            |            |            |     |     |     |     |     | 1233 |
| TGATCACTAA | AAAAAAAAAA  | AAAAAAAAAA | AAAAAAAAAA   | AAAAAA      |             |     |     |            |            |            |     |     |     |     |     | 1279 |

## (2) INFORMATION FOR SEQ ID NO:32:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 238 amino acids

-43/43-

(B) TYPE: polypeptide  
 (C) STRANDEDNESS: N/A  
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: Protein

(A) NAME/KEY: Ptilosarcus gurneyi Green Fluorescent Protein (GFP)  
 (B) LOCATION:  
 (D) OTHER INFORMATION: Ptilosarcus Green Fluorescent Protein (GFP)

(vi) ORIGINAL SOURCE: Ptilosarcus gurneyi

(x) PUBLICATION INFORMATION

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

```

Met Asn Arg Asn Val Leu Lys Asn Thr Gly Leu Lys Glu Ile Met Ser
1           5           10           15
Ala Lys Ala Ser Val Glu Gly Ile Val Asn Asn His Val Phe Ser Met
          20           25           30
Glu Gly Phe Gly Lys Gly Asn Asn Val Leu Phe Gly Asn Gln Leu Met Gln
          35           40           45
Ile Arg Val Thr Lys Gly Gly Pro Leu Pro Phe Ala Phe Asp Ile Val
          50           55           60
Ser Ile Ala Phe Gln Tyr Gly Asn Arg Thr Phe Thr Lys Tyr Pro Asp
65           70           75           80
Asp Ile Ala Asp Tyr Phe Val Gln Ser Phe Pro Ala Gly Phe Phe Tyr
          85           90           95
Glu Arg Asn Leu Arg Phe Glu Asp Gly Ala Ile Val Asp Ile Arg Ser
          100          105          110
Asp Ile Ser Leu Glu Asp Asp Lys Phe His Tyr Lys Val Glu Tyr Arg
          115          120          125
Gly Asn Gly Phe Pro Ser Asn Gly Pro Val Met Gln Lys Ala Ile Leu
          130          135          140
Gly Met Glu Pro Ser Phe Glu Val Val Tyr Met Asn Ser Gly Val Leu
145          150          155          160
Val Gly Glu Val Asp Leu Val Tyr Lys Leu Glu Ser Gly Asn Tyr Tyr
          165          170          175
Ser Cys His Met Lys Thr Phe Tyr Arg Ser Lys Gly Gly Val Lys Glu
          180          185          190
Phe Pro Glu Tyr His Phe Ile His His Arg Leu Glu Lys Thr Tyr Val
          195          200          205
Glu Glu Gly Ser Phe Val Glu Gln His Glu Thr Ala Ile Ala Gln Leu
          210          215          220
Thr Thr Ile Gly Lys Pro Leu Gly Ser Leu His Glu Trp Val
225          230

```

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**